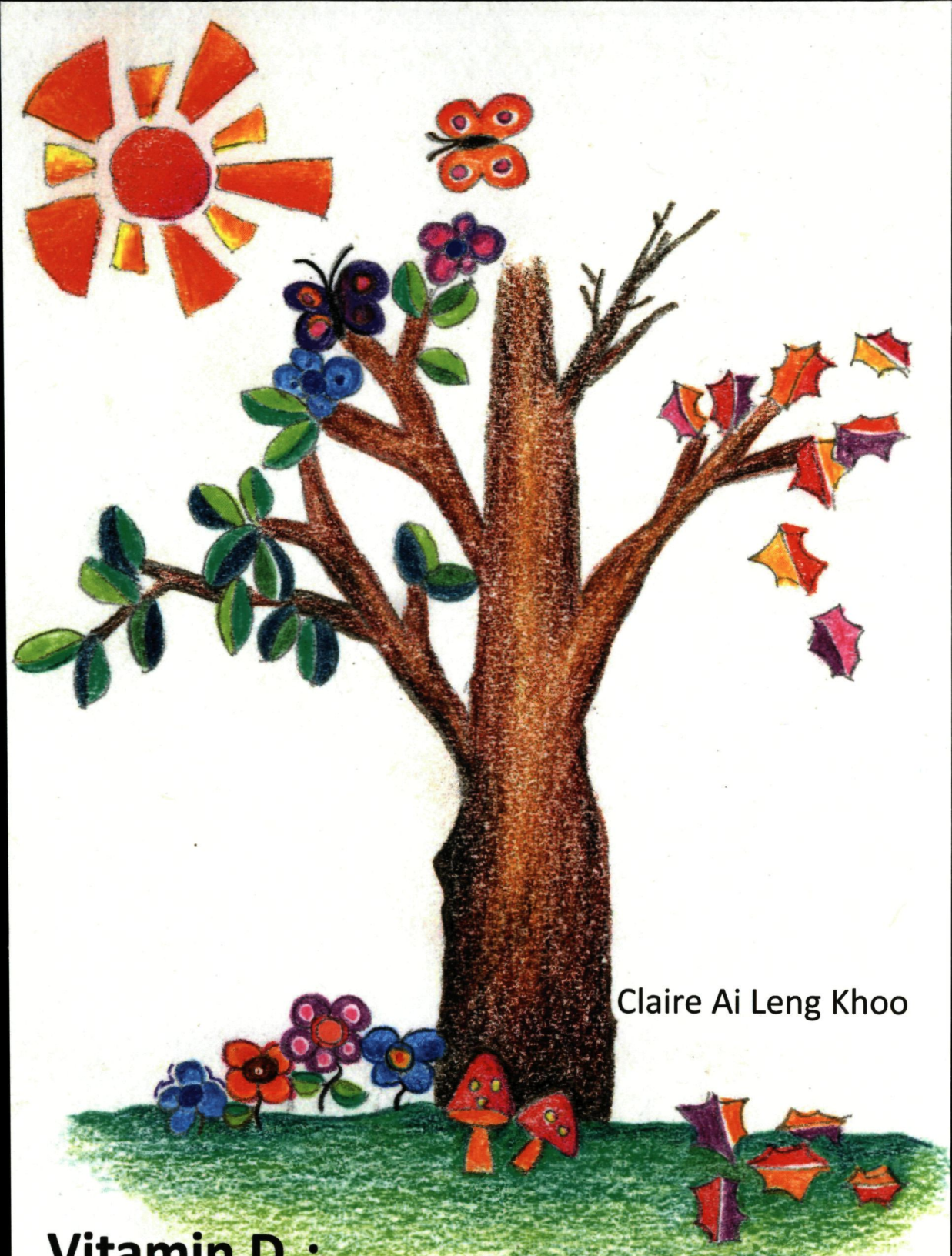

Claire Ai Leng Khoo

Vitamin D_3: Modulator of the Immune Response

Vitamin D_3:

Modulator of the Immune Response

Claire Ai Leng Khoo

The research presented in this thesis was performed at the Department of Laboratory Medicine (Laboratory of Medical Immunology), Department of Medicine, Nijmegen Institute for Infection, Inflammation and Immunity, Radboud University Nijmegen Medical Center, The Netherlands

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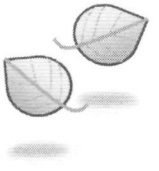
To my three boys

Louis, Clement and Damian

Contents

Introduction



THE BIRTH AND RE-BIRTH OF VITAMIN D_3

Rickets is a bone disease associated with vitamin D_3 deficiency that was first described by Daniel Whistle in 1645. The Cambridge physician Francis Glisson gave a detailed account of rickets in 1650 through his comprehensive investigations (1). However, it was only in the early 20th century that a better understanding of the causative factors of rickets emerged; the disease being attributed to a lack of vitamin D_3. In 1919, Mellanby's theory of rickets resulting from a nutritional disorder (2) had initiated further work that lead to its association with vitamin D_3. In 1922, a major breakthrough was achieved when Harriette Chick showed that whole milk and cod liver oil reduced the prevalence of rickets in children in Vienna (3). By 1930s, the use of cod liver oil in the prevention and treatment of rickets became widespread (4).

During the same period, the discovery of vitamin D_3 and its biosynthesis pathway was elucidated from the work of various researchers in North-Western Europe and North America (Figure 1). In 1914, McCollum and Davis were credited for the discovery of the first accessory food substance to be recognized as vitamin; they called it 'fat-soluble A'. Cod liver oil containing 'fat-soluble A' was first believed to be a single vitamin capable of curing xerophthalmia (eye condition resulting from vitamin A deficiency) and rickets. In 1922, McCollum showed that cod liver oil heated at the temperature of boiling water for 12 to 20 hours retained its antirachitic activity in rats but was no longer effective against xerophthalmia. It was apparent that two factors were present in 'fat-soluble A' and they were later named as vitamin A and vitamin D (5). While McCollum demonstrated that rickets could be treated with cod liver oil, Huldshinsky adopted a new approach by exposing rachitic children to an ultraviolet (UV)-emitting lamp for two months and observing great improvement. This dichotomy was deciphered by Steenbock and Hess who demonstrated that UV light induced vitamin D in the fatty portions of diet and of the skin (6). The bridging of the knowledge that photosynthesized vitamin D and vitamin D in cod liver oil were similar set the platform for a new wave of research leading to the discovery of the biosynthesis pathway of vitamin D_3. In 1926, Hess (from New York) started working with Windaus (from Göttingen) and tested 30 different steroids from various plant sources for antirachitic activity upon radiation. Together with Rosenheim (from London), they managed to identify ergosterol as the cholesterol which can be converted to vitamin D (7). The ultraviolet irradiation product of ergosterol, known as calciferol or vitamin D_2, was chemically characterized in 1931 by Windaus and its structure was established in 1936 (8). Windaus played a major role in the identification, purification and understanding of the chemical properties of vitamin D. He was

awarded the Nobel Prize for chemistry in 1928 for his work on the constitution of sterols (9). In 1955, the steps involved in the photochemical conversion from ergosterol to calciferol (vitamin D_2) was reported in French. The complete sequence of steps, from the conversion of 7-dehydrocholestrol to pre-vitamin D_3 synthesis in the skin, leading to the production of $1,25(OH)_2D_3$ was illustrated by Holick in 1980 (10).

Almost a century after the discovery of vitamin D_3 (The Birth), vitamin D_3 has re-invented its role in human health (The Re-birth). Currently, the research interest concerning vitamin D_3 is focused primarily on its effects on the immune system, cardiovascular system and as anti-tumor therapy; these are often referred to as the non-classical actions. Two pivotal concepts which fuel this heightened interest are: 1) sub-optimal vitamin D status is more common than previously recognised and 2) new perspectives on the non-hormonal impact of its active metabolite, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are known.

Until the late 1990s, the criterion for adequate vitamin D_3 status was simply the absence of rickets or osteomalacia. Circulating serum 25-hydroxyvitamin D_3 ($25(OH)D_3$) concentration has now been accepted as an appropriate marker of vitamin D_3 status. The International Osteoporosis Foundation states that a serum $25(OH)D_3$ concentration greater than 75 nmol/L (30 ng/ml) should be targeted (11). A serum $25(OH)D_3$ concentration of less than 25 nmol/L (10 ng/ml) is defined as deficient, while concentrations less than 50 or 75 nmol/L are generally considered as insufficient. Vitamin D_3 insufficiency is increasingly being recognized as a prevalent health problem and affects an estimated 1 billion people worldwide. Across the world, individuals at risk of vitamin D_3 deficiency include those with dark skin tone, young children, pregnant women and the elderly (12). Vieth has given an interesting insight on human evolution and vitamin D_3 deficiency, with the latter condition leading to natural selection for lighter skin tone (13). Through evolution, clothing and shelter minimize the biosynthesis of vitamin D_3 by the skin. Ultraviolet exposure of the entire skin surface of an adult is equivalent to 10 000 IU vitamin D (14). Exposure of the face and arms constitutes only 5% of the skin surface area. Moreover even for those who live in sunny conditions, they are not assured of a desirable $25(OH)D_3$ concentration due to their way of life as compared to their non-human primate counterparts.

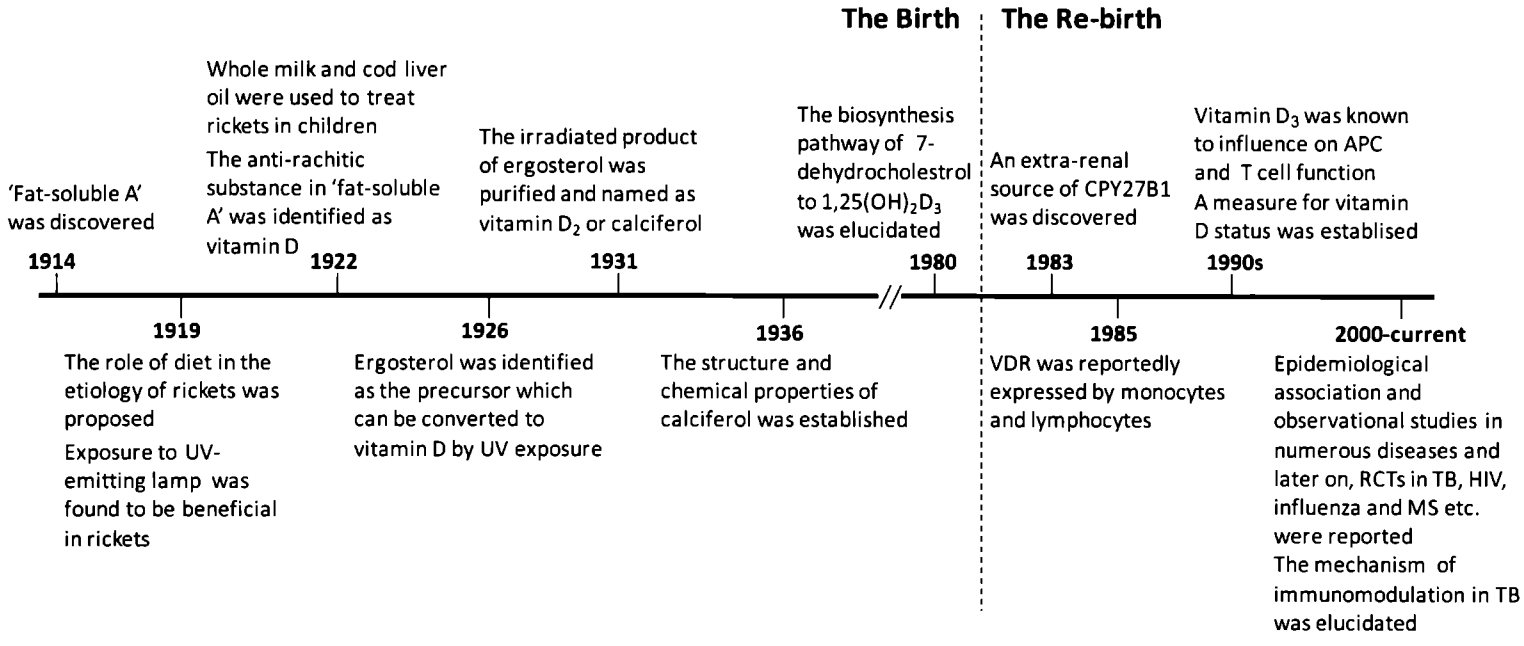


Figure 1. Summary of the significant events leading to the discovery of vitamin D_3 and its non-classical actions

In modern times, people generally stay indoors most of the time due to a shift from agriculture to an industrial and technological society. Moreover, the increased use of sunscreen and decreased outdoor activities may also have contributed to the widespread prevalence of vitamin D_3 insufficiency during the last decades. As mankind migrated out of Africa, the vitamin D_3 store declined because of a reduced ultraviolet exposure at higher latitudes. Skin tone does not impact the amount of $1,25(OH)_2D_3$ being produced, but darker skin tone does require longer exposure. Therefore, vitamin D_3 deficiency favors natural selection for lighter skin tone in geographical areas with lower exposure to ultraviolet light. Epidemiological data have established a relationship between vitamin D_3 deficiency with various health conditions such as infectious diseases (15-18), autoimmune disorders (19), cardiovascular diseases (20) and cancer.

Traditionally, vitamin D_3 is known for its function in regulating calcium and phosphate homeostasis by enhancing their intestinal absorption, and maintaining bone mineralization by mobilizing osteoclastic stem cells to mature and increasing bone resorption (21). A completely novel area of interest emerged in the 1980s when several studies suggested a link between vitamin D_3 and the immune response. One of the initial observations was the extra-renal synthesis of $1,25(OH)_2D_3$ in patients with the granulomatous disease sarcoidosis (22). Vitamin D_3 is first hydroxylated into $25(OH)D_3$ by 25-hydroxylase (CYP27A1) in the liver and further converted by 1α-hydroxylase (CYP27B1) in the kidney into the biologically active form of vitamin D, $1,25(OH)_2D_3$ (21) (Figure 2). Macrophages within the granuloma serve as an extra-renal source of CYP27B1, which is up-regulated by interferon (IFN)γ and lipopolysaccharide (23). Subsequent studies also revealed that $1,25(OH)_2D_3$ synthesis by macrophages is common to granulomatous diseases in general, as well as tumors which involve significant macrophage infiltration (24). Other tissues known to possess the ability to synthesize $1,25(OH)_2D_3$ include pancreatic islets, cerebellum, colonic epithelial cells, breast epithelial cells and keratinocytes. The ability to produce $1,25(OH)_2D_3$ for local regulation of cellular activity is referred to as paracrine effects. This explains why vitamin D_3 is related to a variety of cellular functions and can influence many aspects of human health, as exemplified in Figure 2.

The pioneering work leading to the discovery of vitamin D_3 involvement in the adaptive immune system showed the expression of vitamin D receptor (VDR) by human T lymphocytes (25;26). The VDR is a steroid hormone nuclear receptor pivotal in mediating the cellular effects of vitamin D_3. Activation of the VDR by the active form of vitamin D,

$1,25(OH)_2D_3$ leads to modified gene expression. VDR signaling can influence the expression of more than 200 genes (27). Vitamin D receptor in T cells influence T cell receptor (TCR) signaling, leading to T cell activation (28). VDR polymorphisms have been associated with the clinical course of tuberculosis (29), human immunodeficiency virus (HIV) (30), and viral respiratory tract infections (31).

Over the years, we learn about the interplay between $1,25(OH)_2D_3$ and the immune response through *in vitro* and animal models. We discuss in depth the various immunomodulatory roles of vitamin D_3 in Chapter 2 of this thesis. In brief, $1,25(OH)_2D_3$ modulates the antigen presenting ability and the differentiation of monocytes/macrophages and dendritic cells. It can also shape the phenotype and function of T helper cells and inhibit T cell proliferation (32). Progressively, we begin to understand the extent of which vitamin D_3 can influence human health and its effect on various diseases such as multiple sclerosis, type I diabetes mellitus and more recently, cardiovascular diseases (Figure 2). Based on the knowledge harnessed from studies on the role of vitamin D_3 on the innate and adaptive immune system, observational studies in selected diseases and epidemiology findings, research on its potential clinical applications has gathered tremendous enthusiasm and has progressed towards randomized placebo-controlled trials in recent years (33;34). Having said that, there are new challenges presented to vitamin D immunity research and much still needs to be learned on its specific mechanism of immunomodulation in disease states and its therapeutic benefits.

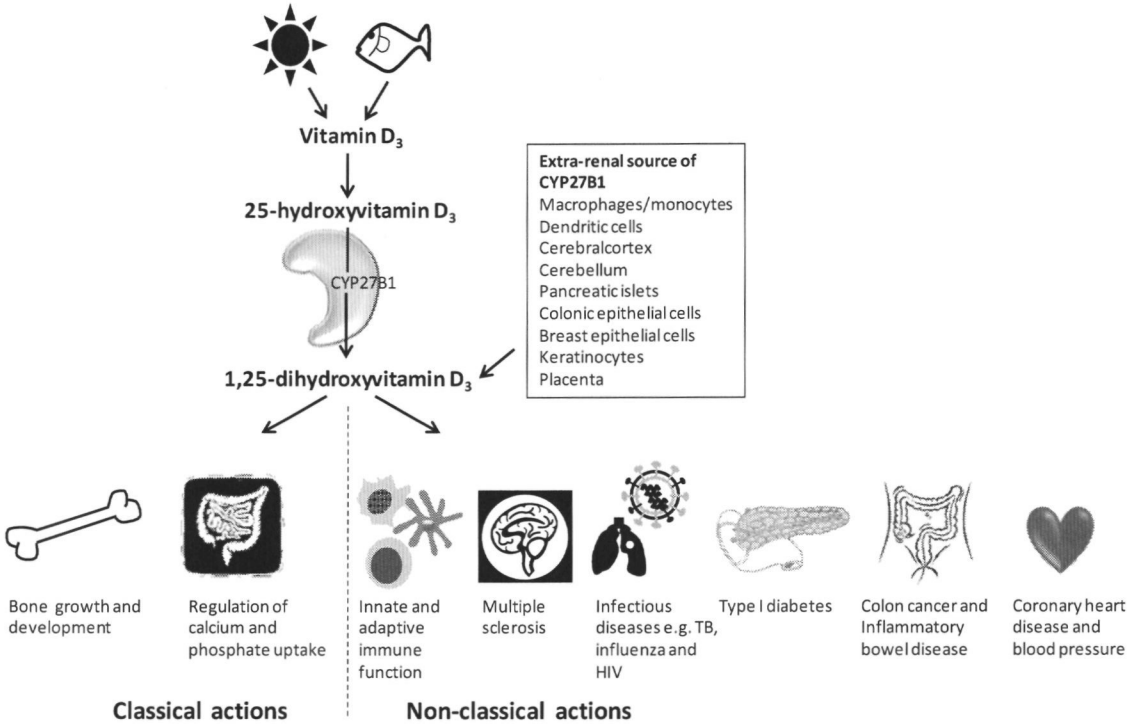


Figure 2. Metabolism of vitamin D_3 and an overview of its classical and non-classical actions

THESIS OBJECTIVES

The aims of this thesis are to explore in greater details the role of vitamin D_3 in both the innate and adaptive immune system, and to contribute towards knowledge on its mechanisms and potential clinical applications in infectious diseases. We first examined its effects on the immune system in the presence of an infection, in order to understand better its clinical role. We also investigated its immunological function on a physiological basis in healthy individuals, and on a cellular basis by focusing on regulatory T cells (Tregs).

The immunomodulatory properties of vitamin D_3 have generated tremendous interest in the field of autoimmune and immune-mediated diseases (32). In **Chapter 2** of this thesis, we reviewed the *in vitro* and clinically relevant data on the role of vitamin D_3 in various infectious diseases, with the intent to identify its therapeutic potential and to identify novel areas for further research.

Vitamin D_3 has been known for a long time to influence *Mycobacterium tuberculosis* (MTB) infection (35-38). In order to improve our understanding on its role in the immune response to MTB, in **Chapter 3** we investigated the effect of $1,25(OH)_2D_3$ on the proinflammatory cytokine production induced by *M. tuberculosis*. We also studied the underlying mechanisms responsible for its effect on cytokine production, as well as its effect on the protective antimycobacterial peptide cathelicidin.

The clinical outcome of other infectious diseases such as influenza (16;39), tuberculosis (15;17) and HIV infection (40;41) has also been associated with vitamin D_3 status. Vitamin D_3 has also been examined as a preventive and therapeutic agent in viral influenza (34;42;43) and tuberculosis (44-47). However, the potential impact of vitamin D_3 on fungal infections in general, and on *Candida albicans* infection in particular, is not known. To explore the role of vitamin D_3 on antifungal immunity, we assessed how *in vitro* $1,25(OH)_2D_3$ modulate the innate immune response of human leukocytes challenged by *C. albicans* in **Chapter 4.**

Having established that $1,25(OH)_2D_3$ has the capacity to modulate proinflammatory cytokine production *in vitro* and that this effect is attributable to the reduced surface expression of

pattern recognition receptors, we investigated its effect *in vivo*. It is not known whether seasonal variation in sun exposure and the resultant physiological vitamin D_3 status (48) can modulate the immune response. In **Chapter 5**, we showed that elevated serum vitamin D_3 concentrations during summer act on the innate immune system resulting in attenuation of proinflammatory cytokine response in healthy individuals. Next, we examined the seasonal impact of vitamin D_3 concentrations on the adaptive immune response in **Chapter 6**. We found seasonal changes in the composition of peripheral T cell and circulating Treg. This knowledge can facilitate our understanding on the amelioration of autoimmune diseases in summer, as well as the proposition if vaccine response may perhaps vary when administered during different seasons.

One of the earliest known effect of vitamin D_3 has been its capacity to inhibit T cell proliferation (49;50). Vitamin D_3 is also known to induce Tregs by rendering antigen presenting cells (APC) tolerogenic (51;52) but its immediate effect on human naturally-occurring Tregs is less clear. In **Chapter 7**, we investigated how $1,25(OH)_2D_3$ can directly affect the proliferation, suppressive function and cytokine production of human naturally-occurring Tregs *in vitro*.

Human immunodeficiency virus infection is characterized by progressive $CD4^+$ T cell depletion and immune dysfunction. Vitamin D_3 deficiency is common among HIV-infected patients as compared to healthy controls (18). The high prevalence of vitamin D_3 deficiency in HIV-infected individuals is probably an unwanted result of highly active anti-retroviral therapy (HAART) (53-56). The enzymes involved in the conversion of $1,25(OH)_2D_3$ are part of the cytochrome (CYP)450 superfamily and anti-retroviral agents are known to inhibit or induce these enzymes. In **Chapter 8**, we investigated whether cholecalciferol (vitamin D_3) supplementation can impact Treg trafficking potential in HIV-infected individuals.

A summary of our findings and conclusions of the thesis are presented in **Chapter 9.**

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Translating the Role of Vitamin D_3 in Infectious Diseases

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ABSTRACT

Vitamin D_3 affects both the innate as well as adaptive immune responses. Epidemiological studies have established that vitamin D_3 deficiency plays an important role in tuberculosis (TB) and viral influenza prevalence as well as susceptibility to active disease in TB. Vitamin D_3 status has been associated with the clinical course of human immunodeficiency virus (HIV) infection and drug interaction with anti-retroviral therapy. This article reviews the immunomodulatory capacity of vitamin D_3 and examines the impact of vitamin D_3 supplementation as a preventive or therapeutic intervention with the intent to uncover its potential therapeutic application in infectious diseases and to identify novel areas for future research. We present a review of randomized, placebo-controlled clinical studies conducted in humans which included assessment of the immune function or clinical outcome as study end points. Current data support vitamin D_3 supplementation as risk-modifying intervention in tuberculosis and viral respiratory tract infection, but the optimal dosage regimen remains to be determined. However, to date the knowledge on its role in fungal infection and sepsis is limited although a potential benefit could be harnessed from its ability to curtail the unrestrained proinflammatory response and therefore prevent excessive collateral tissue damage.

INTRODUCTION

Vitamin D_3 has been conventionally associated with calcium and bone metabolism. It is only in recent years that vitamin D_3 has been recognized as a potent modulator of the immune response. Sources of vitamin D_3 include dietary uptake (primarily fatty fish and cod liver oil) as well as cutaneous previtamin D_3 synthesis from 7-dehydrocholestrol upon ultraviolet (UV)-B (290-315 nm) exposure (1). Subsequently, previtamin D_3 undergoes a spontaneous thermal isomerisation into vitamin D_3 which upon entering the circulation, is first hydroxylated into 25-hydroxyvitamin D_3 ($25(OH)D_3$) by 25-hydroxylase (CYP27A1) in the liver (Figure 1). $25(OH)D_3$ is further converted by 1α-hydroxylase (CYP27B1) in the kidney into the biologically active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Metabolism of $1,25(OH)_2D_3$ is regulated by plasma parathyroid hormone levels as well as serum calcium and phosphorus levels. In turn, 24-hydroxylase converts $1,25(OH)_2D_3$ into its inactive calcitroic acid, thereby limiting excessive vitamin D_3 activity.

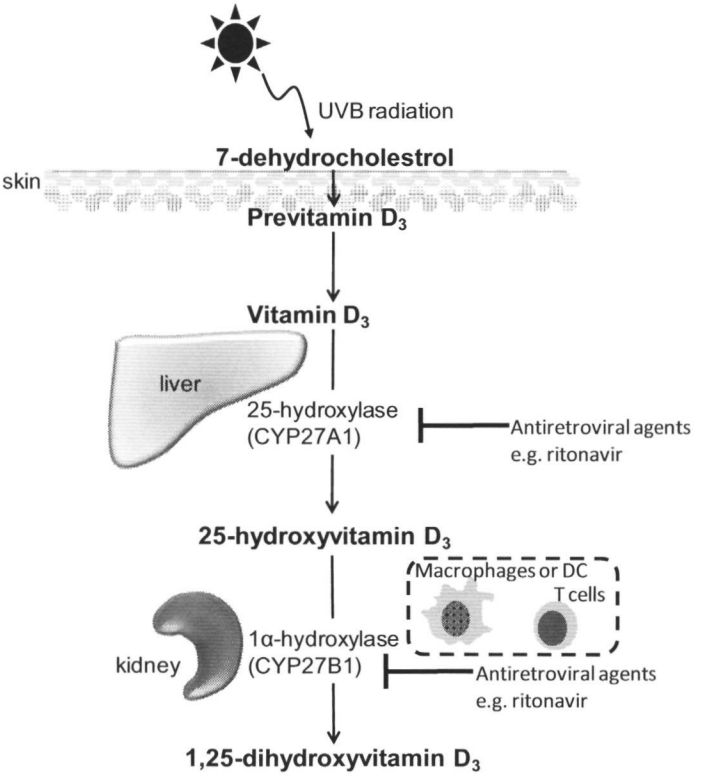

Figure 1. Vitamin D_3 metabolism

Exposure to UVB light (290-315 nm) results in the first step of vitamin D_3 biosynthesis, causing 7-dehydrocholestrol to form previtamin D_3 in the skin. Previtamin D_3 is subsequently converted into

vitamin D_3 which enters the circulation and is first hydroxylated to $25(OH)D_3$ by 25-hydroxylase (CYP27A1) in the liver. $25(OH)D_3$ is further converted by 1α-hydroxylase (CYP27B1) in the kidney into the biologically active form of vitamin D, $1,25(OH)_2D_3$. Human T cells, DCs and macrophages express 1α-hydroxylase and are able to increase cellular $1,25(OH)_2D_3$ levels. 25-hydroxylase (CYP27A1) and 1α-hydroxylase (CYP27B1) belongs to the cytochrome (CY)P450 enzyme superfamily and drugs which inhibit these enzymes can have an impact on vitamin D_3 status.

The vitamin D receptor (VDR) is a steroid hormone nuclear receptor pivotal in mediating the cellular effects of vitamin D_3. Activation of the VDR by the active form of vitamin D, $1,25(OH)_2D_3$ leads to modified gene expression. VDR signaling can influence the expression of more than 200 genes (2). The discovery of VDR expression by human lymphocytes and monocytes was one of the first observations which put forward a role for vitamin D_3 in the immune response (3;4). VDR is expressed by a diverse range of immune cells including macrophages, dendritic cells, neutrophils $CD4^+$ and $CD8^+$ T cells. The receptor is constitutively expressed in monocytes/macrophages (5) while naïve $CD4^+$ T cells display low VDR levels. However, the expression of VDR increases upon activation of T cells, suggesting an important role for vitamin D_3 in influencing adaptive immunity (6;7). T cell antigen receptor (TCR)-mediated signaling via the protein kinase p38 pathway induces up-regulation of VDR, and subsequently induction of phospholipase C (PLC)-gamma1 which is responsible for T cell activation (8).

In this review, we present the *in vitro* immunomodulatory effects of vitamin D_3 when challenged by pathogens; and focus our discussion on the clinical association between vitamin D_3 and its therapeutic potential in various infections such as tuberculosis (TB), viral influenza, human immunodeficiency virus (HIV) and fungal infections. We aim to provide clinicians and researchers with an encompassing view on the prospects of translating knowledge on vitamin D_3 from bench to bedside.

ROLE OF VITAMIN D_3 IN THE INNATE AND ADAPTIVE IMMUNE RESPONSE

Increasing interest and research during the past decade has furthered our understanding on the influence of vitamin D_3 on both the innate and acquired immune response. The host innate immune system mounts an instrumental frontline defence against pathogens. Macrophages and dendritic cells (DCs), which are the primary antigen presenting cells (APC) bridging the innate with the adaptive immune system, represent an important platform through which $1,25(OH)_2D_3$ acts upon. The recognition of microbial pathogen associated

molecular patterns (PAMPs) by pattern recognition receptors (PRRs) leads to APC activation and elicits adaptive immunity. The antigen presenting ability of monocytes/macrophages can be diminished by $1,25(OH)_2D_3$ (Figure 2) as evident by a lower expression of co-stimulatory molecules CD80, CD86 and MHC class-II antigens (9;10). The production of proinflammatory cytokines, such as tumor necrosis factor (TNF)α, interleukin (IL)-1β, IL-6, IL-8 and IL-12 in monocytes, is therefore reduced by vitamin D_3 (11). We and others have shown that this may also be attributable to vitamin D_3's capacity to down-regulate transcription and hence surface expression of PRRs (12-14). Besides limiting the antigen presenting ability of macrophages (9;10), $1,25(OH)_2D_3$ can suppress the maturation and differentiation of DCs and influence cytokine responses in these cells (15). This in turn dictates the type of T cell response. $1,25(OH)_2D_3$ is capable of inducing the differentiation of DCs towards a more tolerogenic phenotype (Figure 2), characterized by a high IL-10 secretion, at the same time with low IL-12 production and a reduced expression of MHC class-II antigens, co-stimulatory molecules CD40, CD80, CD86 and the maturation-induced surface marker, CD83 (16-18). Overall, vitamin D_3 inhibits DC and macrophage functions through attenuation of IL-12 while heightening IL-10-mediated responses. This leads to attenuation of T helper (Th)1 response while maintaining the ability to induce regulatory T cell (Treg) function.

A well-established effect of vitamin D_3 has been its capacity to inhibit T cell proliferation mediated in part through suppression of IL-2 production (19;20), resulting from the direct inhibition of NFATp/AP-1 complex formation (21). Vitamin D_3 can suppress proinflammatory Th1 and Th17 cytokine responses (21-26). While vitamin D_3 down-regulates the Th1 response, at the same time it can enhance IL-4, IL-5 and IL-10 production and promote a Th2 response thereby control the proinflammatory cytokine response. (27-30). Furthermore, $1,25(OH)_2D_3$ either alone or in combination with dexamethasone, can potentiate the differentiation of IL-10-producing $CD4^+CD25^+$ Tregs (31;32). Tregs are responsible for suppressing immune responses and thus limiting excessive inflammation and collateral tissue damage. Previously, this effect of vitamin D_3 has been thought to be mediated through modulation of the APC function exclusively, but recently, Jeffery et al reported that $1,25(OH)_2D_3$ can act directly on $CD4^+CD25^-$ T cells to suppress the production of inflammatory cytokines and generate $Foxp3^+$ Tregs expressing high levels of CTLA-4 which are capable of immune suppression (23). The net result of $1,25(OH)_2D_3$ action on T cell is skewing towards Th2 and Treg differentiation and away from Th1 and Th17 polarization (Figure 2). Thus, these effects underlined a potential role for $1,25(OH)_2D_3$ in the control of autoimmunity.

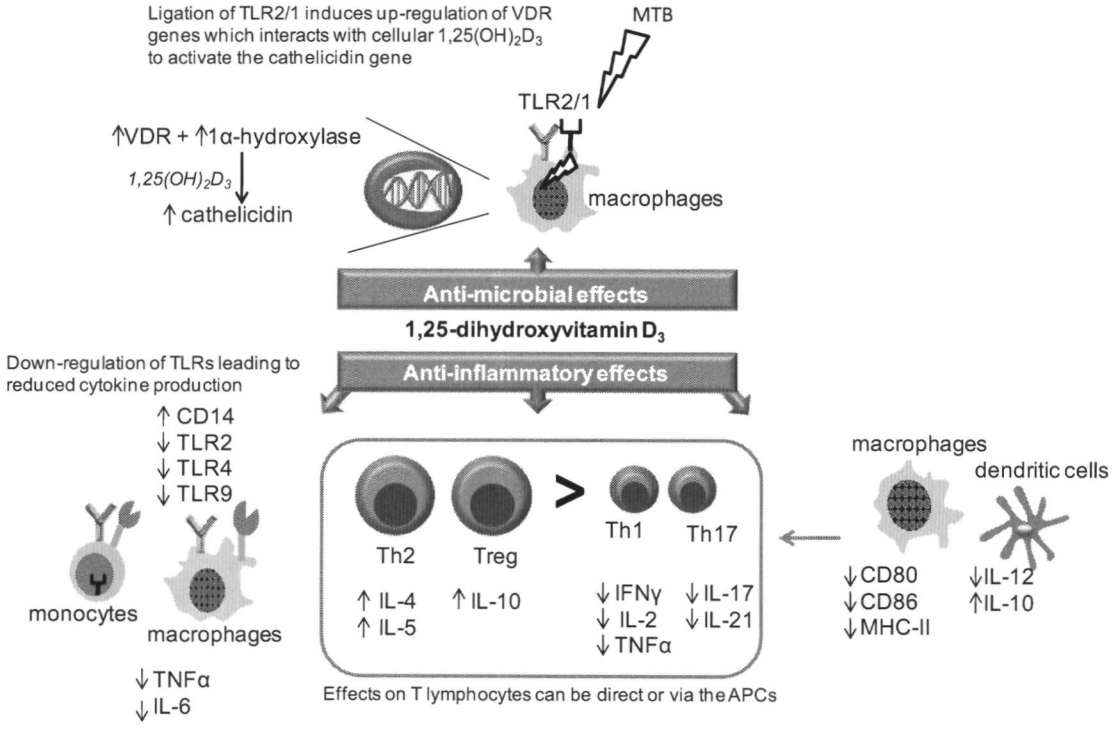

Figure 2. An overview of the anti-microbial and anti-inflammatory effects of vitamin D_3

Vitamin D_3 can influence the differentiation of naïve $CD4^+$ lymphocytes directly and indirectly via the antigen presenting cells (APC). Overall, it favors a T helper (Th)2 and regulatory T cell (Treg) cytokine profile over an anti-inflammatory Th1 and Th17 phenotype. Down-regulation of Toll-like receptor (TLR) 2, TLR4 and TLR9 on human monocytes/macrophages by vitamin D_3 also leads to repression of anti-inflammatory cytokines. The antimicrobial effect of vitamin D_3 upon TB stimulation is triggered when activation of TLR2/1 causes an up-regulation of both vitamin D receptor (VDR) and 1α-hydroxylase genes. The consequential increased in local production of $1,25(OH)_2D_3$ interact with the up-regulated VDR and in turn activated cathelicidin gene and an increased synthesis of cathelicidin, an antimicrobial peptide.

Based on what is known about the effect of vitamin D_3 on the immune system, with an emphasis on proinflammatory Th1 and Th17 responses, this raises the question regarding its modulation of host defence in infections. This is highly relevant as it is thought that an adverse outcome from sepsis may be accentuated by an over-zealous and unchecked proinflammatory host response (33). Hence, we review *in vitro* and clinically relevant data on the role of vitamin D_3 on the immune response when challenged by tuberculosis, sepsis, influenza, HIV and fungal infections, with the intent to identify potential areas of therapeutic application and to uncover novel areas for future work.

EPIDEMIOLOGY OF VITAMIN D_3 DEFICIENCY

25-hydroxyvitamin D_3 is a stable metabolite of vitamin D in the body. The concentration of $25(OH)D_3$ is close to around 1000-fold higher than that of $1,25(OH)_2D_3$. 25-hydroxyvitamin D_3 has a half life of 1-2 months in contrast to that of $1,25(OH)_2D_3$ which is several hours (34;35). As such, $25(OH)D_3$ concentration in the serum is thought to be the best indicator of vitamin D_3 status (34;36). There is still an ongoing debate on what should be the optimal concentration of vitamin D_3. A serum $25(OH)D_3$ concentration of less than 25 nmol/L is considered as deficient, while concentrations between 50 and 75 nmol/L are defined as vitamin D_3 insufficiency (37). Opinions vary when it comes to the optimal vitamin D_3 circulating concentration, though a recent consensus panel recommends that a serum $25(OH)D_3$ concentration greater than 75 nmol/L should be targeted (38), while others suggest that this target concentration should be higher (39). This recommendation is based on the mean serum $25(OH)D_3$ concentration associated with reduction in fracture risk in studies using vitamin D and calcium supplementation. This target $25(OH)D_3$ concentration is also a close estimate for maximal parathyroid hormone suppression and decreasing the likelihood of vitamin D_3 deficiency-induced secondary hyperparathyroidism (40).

Vitamin D_3 deficiency is endemic and thought to affect an estimated 1 billion people worldwide (37). Across the world, individuals at risk of vitamin deficiency include those with dark-skin colour, young children, pregnant women and the elderly (41). Vitamin D supplementation is available in two forms, vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_3 has been found to be more effective in elevating the serum $25(OH)D_3$ concentrations (42). Vitamin D_3 can be administered as low daily doses or in high doses as pulse therapy. The dose response of vitamin D_3 depends on several factors such as baseline serum $25(OH)D_3$ concentration and body mass index (BMI) (43). For a low

baseline $25(OH)D_3$ concentration, a daily dose of 100 IU (2.5 mcg) results in a mean increment of 2.75 nmol/L, while this leads to an increment of 1.75 nmol/L for those with high baseline concentrations (44). The recommended dose for vitamin D_3 supplementation ranges from 800 to 2000 IU daily, though higher dosages are believed to be required for maintaining the target $25(OH)D_3$ concentration of 75 nmol/L (35). At 1000 IU and 4000 IU per day, serum $25(OH)D_3$ concentration > 75 nmol/L was attained in 35% (8/23) and 88% (22/25) of the subjects respectively (45). An increased risk of hypercalcemia may result from excessive vitamin D intake especially during concomitant increased calcium intake or dehydration (35). One important note is that current target serum $25(OH)D_3$ concentrations and dosage recommendations for vitamin D_3 revolves around its application in bone health, while the effective dose required to elicit an effect on the immune system *in vivo* remains to be determined.

VITAMIN D_3 AND TUBERCULOSIS

Tuberculosis remains as one of the most widely spread diseases worldwide. According to the World Health Organization, there were 9.2 million new cases of tuberculosis and 1.7 million deaths in 2007 (46). The earliest clinical application of vitamin D for the management of tuberculosis was documented in 1849 when cod liver oil (rich in vitamin D) was used to treat the disease (47). Subsequently, purified vitamin D_3 in high doses was used to treat skin and pulmonary TB in the pre-antibiotic era before the discovery of effective anti-mycobacterial agents (48-51). Of note, these studies were anecdotal case series and since the mid-1950s, this practice was superseded by the availability of effective anti-tuberculous agents.

Anti-mycobacterial activity is dependent on cell-mediated immune response involving macrophages, T cells and proinflammatory cytokines. Alveolar macrophages are the first line of defence in *Mycobacterium tuberculosis* (MTB) infection. Vitamin D_3 has no direct anti-mycobacterial action and several modes of action have been put forward. Earlier works revealed that $1,25(OH)_2D_3$ induces cellular protection against *M. tuberculosis* in both monocytes (52) and macrophages (53) by limiting the growth of the bacilli. Others have shown that $1,25(OH)_2D_3$ generates a superoxide burst (54) in *M. tuberculosis*-infected cells as an anti-tuberculous defence mechanism. The success of the pathogen lies in its ability to survive and replicate within macrophages to evade detection by the host immune system, in which the avoidance of phagosome-lysosome fusion is vital for *M. tuberculosis* survival.

$1,25(OH)_2D_3$ has been shown to augment phagosome-lysosome fusion via a phosphoinositide 3-kinase signaling pathway in infected macrophages (55).

Recently, Liu et al found that the activation of Toll-like receptor (TLR)2/1 on human monocytes/macrophages by *M. tuberculosis* led to an up-regulation of both 1α-hydroxylase and VDR genes (56). Consequently, this led to increased production of $1,25(OH)_2D_3$, which in turn interacts with the up-regulated VDR and turn on the cathelicidin gene (Figure 2). Cathelicidin is an antimicrobial peptide and the resultant increased cathelicidin production leads to killing of intracellular *M. tuberculosis*. Furthermore, the study showed that when serum concentrations of $25(OH)D_3$ fell below 50 nmol/L, the monocytes/macrophages were not able to initiate this innate immune response. This may provide the basis as to why dark-skinned individuals, who are more prone to being vitamin D_3 deficient, are at higher risks of contracting tuberculosis and tend to have a more aggressive form of the disease (57).

The PAMP-PRR interaction between pathogen and host immune cells is critical for the activation of immune response against bacterial and fungal infections. TLRs 2, 4, 9 and C-type lectin receptor Dectin-1 are involved in recognition of *M. tuberculosis* (58). $1,25(OH)_2D_3$ suppresses expression of TLRs 2, 4 and Dectin-1 on monocytes (13;14). Subsequently, production of the proinflammatory cytokines IL-6, TNFα and interferon (IFN)γ was attenuated while IL-10 was increased. We found that this down-regulation of proinflammatory cytokines resulted from upstream inhibition of TLRs 2, 4 and Dectin-1 transcription and membrane expression, which was reversed by VDR inhibition (59). Furthermore, this observed down-modulation of PRRs was accompanied by increased cathelicidin production. Focusing on the effects of vitamin D_3 on intracellular TLRs, Dickie et al reported that TLR9 expression on monocytes was down-regulated by $1,25(OH)_2D_3$ while TLR3 was unaffected (12). The implication of such down-regulation of proinflammatory cytokine response in the pathogenesis of TB needs to be further studied in lieu of other interactions between $1,25(OH)_2D_3$ and cathelicidin (Figure 2). These two effects may have beneficial consequences, by reducing the collateral tissue damage induced by proinflammatory cytokines, while the antibacterial effects of cathelicidin are enhanced.

Epidemiological data have established that vitamin D_3 deficiency plays a role in TB prevalence and susceptibility to active disease (60-65). Patients with active TB have lower serum vitamin D_3 concentrations as compared to healthy controls according to a meta-

analysis (63). Likewise vitamin D_3 deficiency seems to be associated with a 5-fold higher risk of progression to active TB in household contacts (64). Amongst African immigrants, there was a strong association between vitamin D_3 deficiency and TB infection (60). Serum 25(OH)D_3 concentration below 25 nmol/L was reported in 78% of the patients with TB or history of TB and 33% of those with latent TB infections.

Parallel to the uncovering of new evidence pertaining to the immune-modifying effect of vitamin D_3, there has been a rekindled interest on the possible therapeutic role of vitamin D_3 in TB. Several prospective randomized studies evaluating its application in conjunction with standard TB treatment have been performed (Table 1). An earlier study was carried out in 24 newly diagnosed tuberculous children (age 1.5-13 years old) from Egypt, treated with rifampicin, isoniazid and streptomycin either alone or with 1000 IU (25 mcg) vitamin D for 8 weeks. There were no statistically significant clinical benefits of vitamin D_3 in terms of clinical and radiological responses (66). In another study cohort of 67 adults with pulmonary TB from Indonesia, a daily dose of vitamin D 10,000 IU (250 mcg) was administered for 6 weeks as adjunctive therapy (67). Consequently sputum conversion to negative for acid fast bacteria was 100% in the vitamin D-supplemented group and 77% in the placebo arm. In addition radiological improvement was reported as 88% and 65% in the treatment and placebo group respectively, though this was not statistically significant. Of note, the actual vitamin D_3 concentrations were not measured and hence efficacy of this dose in correcting any underlying deficiency was unknown. On the other hand, a clinical study involving 356 adults from West Africa showed that vitamin D_3 convey neither benefit in clinical outcome (measured by TBscore) nor reduction in mortality at 12 month (secondary endpoint) in both HIV-1 seropositive and seronegative patients (68). The authors employed a high dose pulsed therapy consisting of 100,000 IU (2.5 mg) cholecalciferol given at 0, 5 and 8 months and attributed the lack of therapeutic response to a suboptimal dosing regimen. When pulsed therapy of 100,000 IU cholecalciferol was administered as adjunctive therapy at 0, 14, 28 and 42 days upon initiation of anti-mycobacterial therapy, the mean serum 25(OH)D_3 concentration was significantly increased from 21 nmol/L to 101 nmol/L at day 56 in the intervention group (69). However the time to sputum culture conversion was not affected in the entire treatment cohort though it did significantly enhance sputum culture conversion in individuals with the *tt* genotype of the *Taq*I VDR polymorphism.

To assess the value of vitamin D_3 in preventing reactivation of latent TB, Martineau et al carried out a double-blind randomized trial in 192 healthy TB contacts. They demonstrated

Reference; study design; location	Patients	Vitamin D dosage	Antimicrobial therapy	Therapeutic response	Vitamin D status
Adjunctive therapy					
Morcos et al, 1998; RCT, Egypt	24 children (1 5-13 yo) with pulmonary and extrapulmonary TB	PO 1000 IU daily for 8 weeks	Rifampicin, isoniazid and streptomycin	No statistically significant response	No significant difference between treatment and placebo arm
Nursyam et al, 2006, RCT, Indonesia	67 adults (15-59 yo) with pulmonary TB	PO 10,000 IU daily for 6 weeks	Rifampicin, isoniazid, ethambutol and pyrazinamide	Higher sputum conversion rates ($p < 0\ 05$) and radiological improvement ($p > 0\ 05$)	Not reported
Wejse et al, 2009, RCT, Africa	365 adults (mean 13 yo) with pulmonary and extrapulmonary TB	PO 100,000 IU vitamin D_3 at 0, 2 and 5 month	Rifampicin, isoniazid, ethambutol and pyrazinamide	No benefit in TBscore or reduction in 12-months mortality rate	No significant difference between treatment and placebo arm at 2 months
Martineau et al, 2011, RCT, UK	146 adults (25-42 yo) with pulmonary TB	PO 100, 000 IU vitamin D_3 at 0, 14, 28 and 42 days after initiation of TB treatment	Rifampicin, isoniazid, ethambutol and pyrazinamide	No difference in time to sputum culture conversion except in patients with *tt* genotype of the *Taq*I VDR polymorphism	Mean serum 25(OH)D_3 at day 56 was significantly increased from 21 nmol/L to 101 nmol/L
Preventive therapy					
Martineau et al, 2007, RCT, UK	192 healthy adults TB contacts (median 30 yo)	PO 100,000 IU vitamin D_2 stat	None	Enhanced cellular immunity against TB in-vitro	Induced 91% increase in serum 25(OH)D_3 and corrected deficiency for 6 weeks

Table 1. Clinical studies on vitamin D_3 supplementation in *Mycobacterium* infections

that a single dose of 100,000 IU (2.5 mg) ergocalciferol (vitamin D_2) corrected the deficiency and resulted in enhanced immunity to mycobacterial infection *in vitro* (70). Vitamin D_3 enhanced the ability of the whole blood to restrict BCG-lux luminescence *in vitro*, which corresponded to a beneficial effect of vitamin D_3 on restricting the growth of bacillus Calmette-Guerin. Overall, vitamin D_3 supplementation showed promises as a risk-modifying agent in TB contacts (70). There are also *in vitro* evidence that vitamin D_3 enhance cellular immunity against *M. tuberculosis* (56). Even though chemotherapy has replaced the use of pharmacological doses of vitamin D_3 for the treatment of TB, there is indicative evidence for use of vitamin D_3 in the management of this disease. This is especially true for certain TB endemic regions where populations may have a high prevalence of vitamin D_3 deficiency due to their skin pigmentation (60;64;65). Moreover, some of these areas are also poor in resources and vitamin D_3 supplementation is an attractive approach to improve their vitamin D_3 status due to its relatively low cost. However, the therapeutic dosage and clinical benefits of adjunctive vitamin D_3 in TB treatment remains to be determined.

VITAMIN D_3 AND SEPSIS

A role for vitamin D_3 in the realm of sepsis pathogenesis and sepsis-induced disseminated intravascular coagulation (DIVC) may be proposed due to its ability to modulate cytokine responses. In the setting of sepsis, it is thought that whilst a 'proinflammatory-type' immune response is desirable initially, an unnecessarily prolonged 'hyperinflammatory phenotype' may be adverse for the outcome. When unchecked, an overzealous host inflammation response results in cellular and organ damage (33). The capacity of $1,25(OH)_2D_3$ to modulate cytokine response and induce antimicrobial cathelicidin production makes it a promising adjuvant to treatment of sepsis.

There have been several studies which examined the role of vitamin D_3 in experimental sepsis models. Sadeghi et al demonstrated *in vitro* that $1,25(OH)_2D_3$ down-regulates TLR2 and TLR4 expression on monocytes, resulting in impaired TNFα production (13). During sepsis, lipopolysaccharide (LPS), a TLR4 ligand from Gram-negative pathogens induces TNFα and triggers release of tissue factor (TF). This results in thrombosis and consequently, DIVC. The modulatory role of $1,25(OH)_2D_3$ on TLR4-mediated effects limits the release of TF and this function is conceivably beneficial. In animal models, $1,25(OH)_2D_3$ treatment was associated with improved blood coagulation parameters in relation to DIVC during LPS-induced sepsis (71-73). Asakura et al pointed out that this protective effect of vitamin D_3 in

modulating coagulation disorders is specific to LPS-induced DIVC but not TF-induced DIVC (71). As seen in other settings, proinflammatory cytokines such as IL-12 and IFNγ were markedly reduced by vitamin D_3 treatment in LPS-induced sepsis while IL-10 production was up-regulated; a higher survival rate was seen in the vitamin D_3-treated rats (74). It is also interesting to note that treatment with $1,25(OH)_2D_3$ can inhibit LPS-induced activation and vasodilatation of the vascular endothelium in rats (75).

Besides these *in vitro* and animals studies, one recent study reported a relationship between serum $25(OH)D_3$ concentrations and cathelicidin levels. Jeng et al reported that lower serum $25(OH)D_3$ and vitamin D binding protein concentrations were seen in critically ill patients as compared to healthy controls. More than 95% of the critically ill subjects with and without sepsis were found to be vitamin D_3 deficient (defined as $25(OH)D_3 < 30$ ng/ml or 75 nmol/L), while that number was 60% for the healthy controls. A lower systemic level of cathelicidin was found to be associated with lower serum $25(OH)D_3$ concentrations (76).

VITAMIN D_3 AND VIRAL RESPIRATORY TRACT INFECTIONS

It has been estimated that in a year, at least one upper respiratory tract infection (URTI) afflicts 72% of adults, of which viruses represent the most common pathogens. The main endogenous source of vitamin D_3 in the body derives from biosynthesis in the skin upon ultraviolet exposure (77). A seasonal variation in vitamin D_3 status in the temperate climates has been recorded. There has been a concern regarding the risk of vitamin D_3 insufficiency among populations residing at elevated latitudes where solar radiation during a significant part of the year is inadequate for endogenous vitamin D_3 synthesis (41). Seasonality in influenza and URTI occurrence has been attributed to low wintertime vitamin D_3 levels (78-80). Recently an inverse association between serum $25(OH)D_3$ concentrations and incidence of URTI has also been demonstrated. A significant reduction in the risk of developing viral URTI was attributed to serum $25(OH)D_3$ concentrations exceeding 75 nmol/L (81). In other studies, this benefit was conferred by a higher $25(OH)D_3$ concentration of 100 nmol/L (79;82).

Currently, clinical studies evaluating the potential benefits of vitamin D_3 supplementation in reducing the occurrence of seasonal influenza in adults have not been conclusive (83;84), although it seems to be associated with protective benefits in children (85) (Table 2). In a

double-blind randomized controlled trial (RCT) involving 167 school children (age 6-15 years old), supplementation with 1200 IU cholecalciferol daily during winter significantly reduced the incidence of influenza A infections On the other hand, supplementation with cholecalciferol in 164 adult males at a daily dose of 400 IU for 6 months (October to March) did not reduce the incidence of URTI as determined by the number of days absent from work. This could be explained by an inadequacy in the dosage used leading to a lack of effect on the serum $25(OH)D_3$ concentration with only 29% individuals in the intervention group attaining concentrations beyond 80 nmol/L (mean 72 nmol/L) (84). In a post hoc analysis, Aloia et al found a decline in the rates of self-reported influenza and cold symptoms in patients supplemented with vitamin D_3 (86). The same group later attempted to validate this effect in a randomized study and found that while daily supplementation of 2000 IU cholecalciferol significantly increased the mean serum $25(OH)D_3$ concentrations to 88.5 nmol/L after 12 weeks, there was no benefit in reducing the severity and incidence of URTI during winter (83). Not unexpectedly, GM-CSF, IFNγ, IL-4, IL-8 and IL-10 levels were lower in the intervention group as compared to those in the placebo group (87).

It would be worthwhile to consider conducting randomized placebo-controlled trials in larger populations and with higher doses of vitamin D_3 to explore the preventive role of vitamin D supplementation in seasonal influenza and acute URTI. Even though the plausible benefit of vitamin D_3 on influenza and URTI is largely based upon epidemiological data, this hypothesis is also built on a reasonably sound scientific basis. Our group has recently found a seasonal variability in host cytokine responses, most likely due to differences in vitamin D_3 concentrations, which further supports this hypothesis *in vivo* (88). Serum $25(OH)D_3$ concentrations were higher during summer relative to winter, which correlated with a down-regulation of proinflammatory cytokine production. Putting forward this notion, it is conceivable that vaccination program may confer differential efficacy depending on vitamin D_3 status and (seasonal) environmental UV exposure. Chadha et al conducted a clinical study on prostate cancer patients and found that higher serum $25(OH)D_3$ concentrations were associated with better serological response to influenza vaccine (89). Unfortunately, the $25(OH)D_3$ concentration defining such a response is not known. Conversely, a clinical trial in 175 healthy volunteers showed that co-administration of 40 IU vitamin D_3 with influenza vaccine did not affect serological response (90). Further research is certainly needed to validate this postulation as it will have far-reaching implications for implementation of preventive health policies.

Reference; study design; location	Patients	Vitamin D dosage	Antimicrobial therapy	Therapeutic response	Vitamin D status
Adjunctive therapy in human immunodeficiency virus infections					
Arpadi et al, 2009, RCT, USA	56 HIV-seropositive children (6-16 yo)	PO 100,000 IU vitamin D_3 bi-monthly for 12 months	Antiretroviral therapy	No effect on CD4 counts and viral load	44% achieve serum (trough) 25(OH)D_3 level > 75 nmol/L
Preventive therapy in viral URTI and influenza					
Urashima et al, 2010, RCT, Japan	167 children (6-16 yo)	PO 1200 IU vitamin D_3 daily for 4 months (in winter)	None	Significant reduction influenza A infections (relative risk 0 58, 95% CI 0 34, 0 99)	Not reported
Laaksi et al, 2010, RCT, Finland	164 adult males (18-28 yo)	PO 400 IU vitamin D_3 daily for 6 months	None	No benefit in reducing occurrence of URTI	Inadequate effect with only 29% achieve serum 25(OH)D_3 level > 80 nmol/L
Li-Ng et al, 2009, RCT, USA	162 adults (mean 58 yo)	PO 2000 IU vitamin D_3 daily for 12 weeks	None	No benefit in reducing occurrence of URTI in winter	73% achieve serum 25(OH)D_3 level > 75 nmol/L

Table 2. Clinical studies on vitamin D_3 supplementation in viral infections

VITAMIN D_3 AND HUMAN IMMUNODEFICIENCY VIRUS INFECTION

Human immunodeficiency virus infection is characterized by progressive $CD4^+$ T cell depletion and immune dysfunction. The capacity of $1,25(OH)_2D_3$ to modulate HIV infection has been examined in human monocytes/macrophages and yielded controversial results. Viral replication was reportedly either inhibited or enhanced following pre-treatment with $1,25(OH)_2D_3$ *in vitro* (91-93). Conflicting data also arises as to whether addition of $1,25(OH)_2D_3$ subsequent to HIV infections would result in inhibition (94) or enhancement (95) of viral replication. To date, this issue remains largely unresolved. However, it has been suggested that CD4 surface expression on monocytes could be reduced by $1,25(OH)_2D_3$ (91;92) and this may constitute a mechanism to control viral entry and slow disease progression.

In addition, it is known that chemokine receptors CCR5 and CXCR4 act together with CD4 as co-receptors for viral entry (96). $1,25(OH)_2D_3$ is able to influence lymphocytic homing and chemokine receptors expression on $CD4^+$ lymphocytes. In one study involving human leukemic cell line, the surface expression of CXCR4 was enhanced by $1,25(OH)_2D_3$ (97). This effect was specific to the cell clone which sustains poor HIV replication. It remains to be tested whether $1,25(OH)_2D_3$ can also modulate CCR5 and CXCR4 expression on HIV target cells thereby altering the course of viral replication.

Serum $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations are often decreased among HIV-infected patients as compared to healthy controls (98). Low levels of $1,25(OH)_2D_3$ and VDR polymorphism have been associated with low CD4 counts, immunological hyperactivity, AIDS progression and HIV-related mortality (99-102). In a Norwegian (n=53) and a Tanzania (n=884) cohort of HIV-infected patients, higher vitamin D_3 status offered protection against disease progression and mortality (101;102). The high prevalence of vitamin D_3 deficiency in HIV-infected individuals (98) is probably an unwanted result of highly active anti-retroviral therapy (HAART) (103-106). The enzymes involved in the conversion of $1,25(OH)_2D_3$ are part of the cytochrome (CY)P450 superfamily and anti-retroviral agents are known to inhibit or induce these enzymes. Thus, the interaction between HAART and vitamin D_3 metabolism enzymes of CYP450 superfamily offers a likely explanation to this observation. In a study involving HIV-infected subjects, serum $1,25(OH)_2D_3$ concentrations was found to be the lowest in patients receiving protease inhibitors (PI) as part of their HAART (105). The postulation that PI suppresses the activity of 1α-hydroxylase was validated in an *in vitro*

study: in human hepatocyte and monocyte cell lines which express 25-hydroxylase and 1α-hydroxylase respectively, the PI-treated cells showed a markedly reduced vitamin D_3 metabolic capacity (107). Recently, other antiretroviral agents such as zidovudine (nucleoside reverse transcriptase inhibitor), efavirenz and nevirapine (non-nucleoside reverse transcriptase inhibitor) have also been reported to reduce serum vitamin D_3 concentrations (103;104;108). Therefore, the complexity of disease and drug interference with vitamin D_3 availability justifies careful monitoring and correction of vitamin D_3 status in this group of patients.

The clinical effects of vitamin D_3 has not been widely documented in HIV- infected patients since there have been very few studies performed using vitamin D_3 supplementation alone in this group of patients (Table 2). In a cohort of 56 children and adolescents (age 6 to 16) randomized to receiving a bi-monthly supplementation of 100,000 IU cholecalciferol (with daily doses of calcium) or placebo, no differences in CD4 count or viral load over a 12-month period were observed, although serum $25(OH)D_3$ concentrations were higher in the treated group (109).

VITAMIN D_3 AND FUNGAL INFECTIONS

Half a century ago, vitamin D_3 was used in the treatment of chromoblastomycosis (110) and this treatment was repeated recently (111). We have recently assessed the effect of $1,25(OH)_2D_3$ on the anti-fungal host defence against a *C. albicans* model (14). $1,25(OH)_2D_3$ down-modulated the proinflammatory Th1 and Th17 cytokine responses when human leukocytes are stimulated with *C. albicans*. This effect is mediated by suppression of TLR2, TLR4, Dectin-1 and mannose receptor transcription, leading to reduced receptor expression. The above observations were validated *ex vivo* in healthy volunteers followed through the four seasons of the year in concordance to the fluctuating vitamin D_3 levels. Besides providing an insight on the role of vitamin D_3 on the pathophysiology of *Candida* infections, these also present *in vivo* evidence of differential immunomodulatory effects mediated by the seasonal fluctuation of vitamin D_3 levels.

In a similar study performed on $CD4^+$ T lymphocytes isolated from *Aspergillus fumigatus*-colonized cystic fibrosis patients, low serum vitamin D_3 concentrations were associated with a heightened Th2 activity in patients with allergic bronchopulmonary aspergillosis (112). *In*

vitro, vitamin D_3 reduced OX40 ligand expression in DCs, while tissue growth factor (TGF)β expression was enhanced, resulting in a preferential Treg induction over Th2 lymphocytes.

This suggests a role for vitamin D_3 in the induction of tolerance, and these effects could potentially prevent allergic bronchopulmonary aspergillosis which is caused by a dominant Th2 response. Both these studies suggest important immunomodulatory effects of vitamin D_3 on antifungal immunity and more work needs to be performed to study in greater details how vitamin D_3 might affect the host response in fungal diseases and its potential therapeutic usage.

FUTURE DIRECTIONS

In light of its diverse immunomodulatory functions, we propose several situations related to infections in which vitamin D_3 could be a viable pharmacological adjunct and could modify the course of infections (Figure 3). By virtue of its ability to promote regulatory immune responses, it is reasonable to think of vitamin D_3 as an option to retard the risk of reactivation of latent infections such as tuberculosis. Higher serum 25(OH)D_3 concentrations have been associated with a lower risk of active infection in TB contacts (64). Moreover, tuberculosis is known to be endemic in mostly resource-poor areas such as South-East Asia and Africa, where the inhabitants are primarily of dark skin colour. Given its relative low cost, vitamin D_3 supplementation is an attractive and simple intervention that can potentially reduce the risk of TB progression.

Vitamin D_3 has the ability to reduce inflammatory response in the respiratory tract epithelium (113), and thus provide an attractive pharmacological option to limit disease severity, thereby resulting in less symptoms during acute epidemic viral infections caused by respiratory syncytial virus and influenza virus. Notably, cutaneous biosynthesis of vitamin D_3 is subjected to seasonal variability and wintertime vitamin D_3 deficiency may be one factor associated with the remarkable seasonality of epidemic influenza. With the widespread annual occurrence of seasonal influenza which translates into loss of working days and productivity, it is worthwhile investigating the value of vitamin D_3 supplementation for influenza prophylaxis and for implementing preventive health policies. On a separate note, it is tempting to hypothesize whether response to vaccination efficacy would differ with adjunctive vitamin D_3 therapy given that very little is known at the moment about this

important therapeutic aspect (89;90). Suggestively, vitamin D_3 has a role in mucosal immunity (114), therefore the route of vitamin D_3 administration should be taken into consideration together with that of the vaccine, as there may be distinct roles of vitamin D_3 for vaccines administered subcutaneously or orally.

The counter-inflammatory Th2/Treg profiles induced by $1,25(OH)_2D_3$ (23;26-28;30) may be beneficial as an adjunct immunomodulatory agent in specific circumstances in which dysregulated host immune responses driven by Th1 and Th17 are implicated in the pathogenesis, such as in autoimmune diseases (115;116) and sepsis (33). Nonetheless, one needs to be mindful that in the context of sepsis, mounting an appropriate host proinflammatory response at the onset of infection is vital. It is mainly the subsequent inability to quench an overzealous inflammatory response which may be detrimental to the host. Hence, in lieu of the immune kinetics in sepsis, the use of vitamin D_3 as an immune modulating agent will need to be carefully timed so as to appropriately alter the disease course and optimise the clinical outcome. On another perspective, we postulate that this counter-inflammatory effect of vitamin D_3 would be desirable in chronic infections whereby prolonged inflammatory process contributes to the pathology, as in the case of chronic hepatitis B and hepatitis C infections. Low serum $25(OH)D_3$ concentrations, resulting from reduced vitamin D_3 metabolizing enzyme (CYP27A1) caused by hepatic necroinflammatory activity, in turn correlated with severity of fibrosis and reduced therapeutic efficacy after standard antiviral treatment (117).

Another novel area of vitamin D_3 research would be to explore a possible role in infection-induced autoimmunity. There has been a strong link between vitamin D_3 and multiple sclerosis (MS), while Epstein-Barr virus infection is a known risk factor for MS (118). Modulation of host response to Epstein-Barr virus infection by vitamin D_3 could be extrapolated to a MS risk-modifying modality (119). In addition, vitamin D_3 might also play a role in immune reconstitution inflammatory syndrome upon initiation of HAART, although this is an area largely unexplored (120). More research will be needed before the above-mentioned therapeutic potentials of vitamin D_3 can be harnessed.

Having said that, the level of $1,25(OH)_2D_3$ required to effectively elicit a immune modulating function remains also largely unknown. One of the pioneering works revealing vitamin D_3 as an immunomodulatory agent found that macrophages are able to metabolize $25(OH)D_3$ into

$1,25(OH)_2D_3$ via the enzyme CYP27B1 (121) (Figure 1). Subsequently, DCs and T cells have been shown to possess such capability as well (122;123). Indeed vitamin D_3 affects many systems and holds potential as adjunctive therapy in various disease processes, but the threshold needed to exert an immunomodulatory function in various tissues is not yet defined. As a matter of fact, it is difficult to assess local $1,25(OH)_2D_3$ concentrations at the tissue level *in vivo*, which is further confounded by the inter-individual variability in CYP450 enzymes polymorphism. Nonetheless, it is necessary to derive a surrogate estimate for the physiological $1,25(OH)_2D_3$ concentrations at the tissue level and employ such concentrations in the *in vitro* settings, so that the value of these studies can be extrapolated to clinical application.

Lastly, an interesting aspect for future work would be the interaction of vitamin D_3 with drug metabolism via the CYP450 superfamily. Vitamin D_3 metabolism hinges on 25-hydroxylase and 1α-hydroxylase which are part of the CYP450 enzymes, and anti-retroviral drugs, for instance, are known to induce or inhibit this enzyme system (107). In view of the variable associations of HIV with metabolic diseases processes, it remains to be determined how the modulation of immune responses at the cellular and tissue level via the vitamin D_3 pathway would be influenced by the administration of HAART.

CONCLUSIONS

Current data support the view that vitamin D_3 supplementation holds promises as risk-modifying intervention in tuberculosis and viral respiratory tract infections, but the optimal dosage regimen remains to be determined. Besides infectious diseases, vitamin D_3 has been implicated in potential risk reduction of autoimmune disease processes and malignancy beyond the scope of this review (118;124;125). Even though results from bench and translational studies have been very promising, more clinical studies and larger population-based epidemiological analyses will need to be conducted in order to validate the therapeutic benefits of vitamin D_3.

We propose that randomized placebo-controlled trials taking into account variation in ethnicity, geographical location and seasonal variability in vitamin D_3 status are needed to effectively elucidate its therapeutic value (Figure 3). Supplementation with at least 2000 IU daily for 8-12 weeks to achieve target serum $25(OH)_2D_3$ levels of 75 nmol/L should be

employed in clinical studies. The underlying vitamin D_3 insufficiency/deficiency associated with certain infections should be corrected in order to further examine the clinical and immunological outcomes. The assessment of immunological functions would be of value in correlating with clinical outcomes. Moving forward, the optimal therapeutic doses and serum $25(OH)D_3$ concentrations required to achieve an optimal impact on these non-classical roles of vitamin D_3 need to be determined.

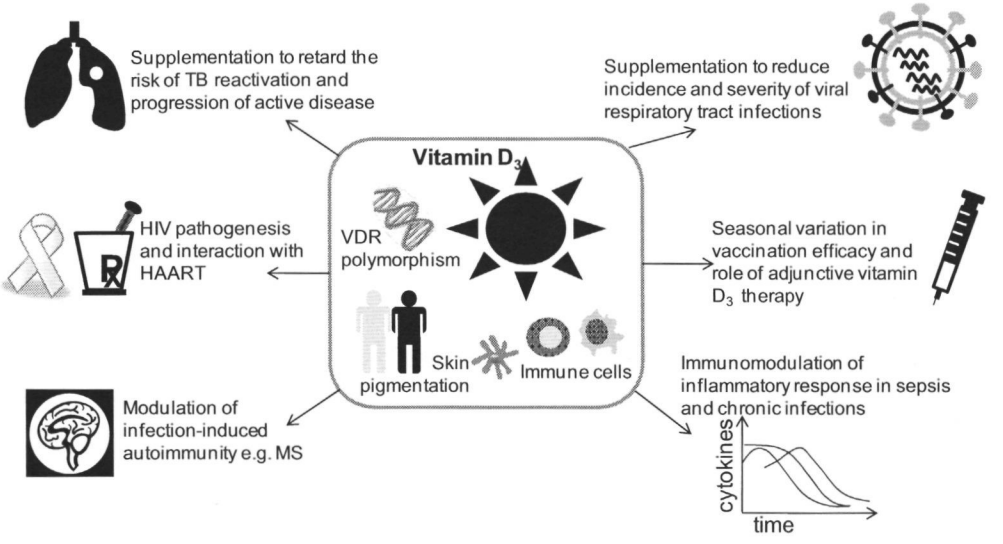

Figure 3. Potential role of vitamin D_3 in infectious diseases

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Vitamin D_3 Down-regulates Proinflammatory Cytokine Response to *Mycobacterium tuberculosis* Through Pattern Recognition Receptors while Inducing Protective Cathelicidin Production

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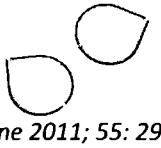

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ABSTRACT

A well-known association between vitamin D_3 and infection with *Mycobacterium tuberculosis* has previously been reported, but little is known regarding the underlying mechanisms. We have investigated how 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) affects the proinflammatory cytokine production induced by *M. tuberculosis*. Furthermore, we explored whether $1,25(OH)_2D_3$ influence the production of the protective anti-mycobacterial peptide cathelicidin. Upon *in vitro* stimulation with *M. tuberculosis*, $1,25(OH)_2D_3$ induced a dose-dependent down-regulation of IL-6, TNFα and IFNγ, while increasing the production of IL-10 in culture supernatant as well as cathelicidin mRNA expression. This effect on cytokine response was not due to modulation of T helper cell differentiation, as T-bet, GATA3, Foxp3 and ROR-γt mRNA expression remained unaffected. Similarly, $1,25(OH)_2D_3$ did not affect suppressor of cytokine signaling (SOCS)1 and SOCS3 mRNA expression. The mechanism whereby $1,25(OH)_2D_3$ inhibited the proinflammatory cytokine response was through reduced expression of the pattern recognition receptors (PRR) such as Toll-like receptor (TLR)2, TLR4, Dectin-1 and mannose receptor, whose mRNA and protein expression were both reduced. The suppression of PRRs could be restored by a VDR antagonist. Upon *M. tuberculosis* stimulation, $1,25(OH)_2D_3$ modulates the balance in cytokine production towards an anti-inflammatory profile by repression of TLR2, TLR4, Dectin-1 and mannose receptor expression, while increasing cathelicidin production. These two effects may have beneficial consequences, by reducing the collateral tissue damage induced by proinflammatory cytokines, while the antibacterial effects of cathelicidin are enhanced.

INTRODUCTION

Mycobacterium tuberculosis (MTB) remains one of the most widespread pathogens. According to the World Health Organisation, there were 9.2 million new cases of tuberculosis (TB) and 1.7 million deaths in 2007 (1). Cod liver oil (2) and subsequently purified vitamin D_3 in high doses were used to treat and prevent TB in the pre-antibiotic era, before the discovery of effective anti-mycobacterial agents in the 1950s (3;4). Vitamin D is either synthesized from previtamin D in the skin during exposure to ultraviolet light, or through dietary intake, such as oily fish (5). Previtamin D is metabolised into 25-hydroxyvitamin D_3 ($25(OH)D_3$) in the liver which is then further metabolised by 1α-hydroxylase in the kidney into its biologically active form, $1,25(OH)_2D_3$. Several studies and meta analysis have established that vitamin D_3 deficiency plays an important role in TB prevalence, and in susceptibility to active disease (6-10).

Emerging evidence on the immunomodulatory properties of vitamin D_3 has offered a better understanding on its effects in mycobacterial infection (11;12). Even though chemotherapy has replaced the use of pharmacological doses of vitamin D_3 for the treatment of TB, vitamin D_3 supplementation is a relatively simple intervention in resource-poor settings where TB is endemic (13). Several clinical studies addressing the efficacy of vitamin D_3 supplementation in TB has been conducted in recent times (14). A trial carried out in TB contacts demonstrated that a single dose of vitamin D_3 corrects deficiency and results in enhanced immunity to mycobacterial infection *in vitro* (15). However, a recent randomized placebo-controlled trial suggested that high-dose pulsed cholecalciferol regimen given with standard TB treatment does not improve clinical outcome or mortality in both human immunodeficiency virus (HIV) seropositive and seronegative patients (16). Thus, identifying the therapeutic role of vitamin D_3 and targeting the right patient cohorts could improve TB treatment outcome.

Anti-mycobacterial activity is dependent on cell-mediated immune response involving macrophages, T cells and proinflammatory cytokines. Alveolar macrophages are the first line of defense in *M. tuberculosis* infection, and T helper (Th)1 responses are essential in mounting an effective immunity against *M. tuberculosis*. T cells, monocytes and macrophages are known to express vitamin D receptor (VDR) (17;18). Moreover, monocytes and macrophages can produce 1α-hydroxylase which converts $25(OH)D_3$ into the biologically active $1,25(OH)_2D_3$ (19;20). Thus, these cells are believed to be important

targets for vitamin D_3 action in TB. The interaction of T cells with infected macrophages is crucial for eliciting protective immunity against the bacillus and depends on the interplay of cytokines released. The aim of this study is to investigate how vitamin D_3 modulates the host cytokine response upon *M. tuberculosis* challenge in human leukocytes, and to assess the involvement of both the innate and acquired immunity. This would offer a better understanding on the role of vitamin D_3 on the immunopathogenesis of TB and create opportunities to explore further its role as an adjunctive therapy in TB.

MATERIALS AND METHODS

Microorganisms

Cultures of *M. tuberculosis* H37Rv were grown to mid-log phase in Middlebrook 7H9 liquid medium supplemented with oleic acid/albumin/dextrose/catalase (Difco, Becton–Dickinson, Palo Alto, California, United States), washed three times in sterile saline, and resuspended in RPMI 1640 medium at the various concentrations. Separate culture suspensions were sonicated for 10 min on ice, in order to obtain cell lysates.

Reagents

TLR2 ligand lipopeptide (S)-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys4-OH, trihydrochloride (Pam3Cys) was purchased from EMC Microcollections (Tübingen, Germany). TLR4 ligand lipopolysaccharide (LPS; Escherichia coli serotype 055:B5) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). An extra purification step of LPS was performed as previously described (21). $1,25(OH)_2D_3$ was purchased from Fluka Biochemika, Sigma–Aldrich (Missouri, USA) and dissolved in absolute ethanol. The 25-carboxylic ester vitamin D analog ZK159222 (22) was synthesized at Bayer Schering Pharma AG (Berlin, Germany) and a stock solution of 10^{-2} M by dissolving in absolute ethanol.

Stimulation assays

Venous blood was drawn into EDTA tubes from healthy volunteers after informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll–Hypaque (Pharmacia Biotech, Uppsala, Sweden). Cells were washed twice in saline, counted and the number adjusted to 5×10^6 cells/ml. A 100 µl volume of PBMC, suspended in culture medium (RPMI 1640 DM; ICN Biomedicals, Costa Mesa, CA) supplemented with 10 µg/ml gentamicin, 10 mM l-glutamine, 10 mM pyruvate and 10% human pooled serum was added to flat-bottomed 96-well plates (Greiner, The Netherlands).

PBMC were pre-incubated with *M. tuberculosis* or RPMI (as unprimed control). Cell cultures were incubated in a 37°C, 95% humidity, 5% CO_2 incubator. The culture supernatants were collected after 24, 48 h or 7 days of incubation as appropriate and stored at -20 °C until cytokine assay.

Flow cytometry

Cells were phenotypically analyzed by five-color flow cytometry (Coulter Cytomics FC 500, Beckman Coulter, Fullerton, USA) using Coulter Epics Expo 32 software. Cells were washed with PBS with 0.2% bovine serum albumin (BSA) before being labeled with fluorochrome-conjugated antibodies (mAb). After incubation for 20 min at room temperature in the dark, cells were washed twice to remove unbound antibodies and analyzed. For cell surface staining, the following mAb were used: Dectin-1 -PE (259931, R&D Systems, Minneapolis, MN), TLR2-FITC (TL2.1) and TLR4-PE (HTA125); both from eBioscience, San Diego, CA and mannose receptor (MR)-FITC (19.2; BD Bioscience, New York, NY).

Cytokine measurements

Interleukin (IL)-6, IL-10 and interferon (IFN)γ concentrations were measured by use of commercial sandwich ELISA kits (Pelikine Compact, CLB, Amsterdam, The Netherlands) according to the manufacturer's instructions. Human tumor necrosis factor alpha (TNFα) was measured by the appropriate commercial ELISA kits (R&D Systems, Minneapolis, MN). Detection limits were 8 pg/ml (IL-6 and IL-10), 20 pg/ml (IL-1β and IFNγ) and 40 pg/ml (TNFα).

Quantitative polymerase chain reaction (PCR)

To determine mRNA expression, RNA was extracted from PBMC stimulated with *M. tuberculosis* in the presence and absence of $1,25(OH)_2D_3$ for 24 h. RNA was extracted from 10^7 PBMC by using 1 ml TRIzol reagent (Sigma, St Louis, MO). Subsequently, 200 µl chloroform and 500 µl 2-propanol (Merck, Darmstadt, Germany) were used to separate the RNA from DNA and proteins. Finally, after washing with 75% ethanol, the dry RNA was dissolved in 50 µl of diethylpyrocarbonate (DEPC) water. The amount and quality of mRNA were determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). cDNA was synthesized from using Superscript Reverse Transcriptase (Invitrogen)

Quantitative real-time PCR was performed using the Bio-Rad iCycler and SYBR green. The following primers were used (5'–3'): TGCCCAGGTCCTCAGCTAC (forward) and GTGACTGCTGTGTCGTCCT (reverse) for cathelicidin; AACCTCAGACAAAGCGTCAAATC

(forward) and ACCAAGATCCAGAAGAGCCAAA (reverse) for TLR2; TTCCTTCAACCAAGAACATAGATC (forward) and TTGTTTCAATTTCACACCTGGATAA (reverse) for TLR4; ACAATGCTGGCAACTGGGCT (forward) and GCCGAGAAAGGCCTATCCAAAA (reverse) for Dectin-1; TCAAGACAATCCACCAGTTACT (forward) and TTCTCTTTGCTGAAATAATACTGGTAGTC (reverse) for MR; GCAGCCGACAATGCAGTCT (forward) and GAACGGAATGTGCGGAAGTG (reverse) for SOCS1; TGCGCCTCAAGACCTTCAG (forward) and GAGCTGTCGCGGATCAGAAA (reverse) for SOCS3 and ATGAGTATGCCTGCCGTGTG (forward) and CCAAATGCGGCATCTTCAAAC (reverse) for β2 microglobulin (B2M) (Biolegio, The Netherlands) as housekeeping gene.

In another system, transcripts were quantified by real-time quantitative PCR on an ABIPRISM 7700 Sequence Detector using pre-designed TaqMan GeneExpression Assays and reagents according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Probes with the following Applied Biosystems assay identification numbers were used: TBX21 (Tbet), Hs00203436_m1; GATA3, Hs00231122_m1; FOXP3, Hs00203958_m1; RORC1-2, Hs00172858_m1; RORC10-11, Hs01076112_m1, using human HPRT1 Endogenous Control (4333768T; Applied Biosystems) as housekeeping gene.

All primers were validated according to the protocol. Mean relative mRNA expression was calculated using Pfaffl method. Values are expressed as ratio of fold increase to mRNA levels of vitamin D_3-untreated cells.

Statistical analysis

Results from at least five sets of experiments were pooled and analyzed using SPSS 16.0 statistical software. Data given as means ± SE and the Wilcoxon signed rank test was used to compare differences between 2 groups: without (control) and with vitamin D_3, unless otherwise stated. The level of significance was set at $P < 0.05$.

RESULTS

$1,25(OH)_2D_3$ diminishes the proinflammatory cytokine response to *M. tuberculosis*

Firstly, we investigated the effects of $1,25(OH)_2D_3$ on immune response to *M. tuberculosis* by stimulating PBMC in the presence of 1, 10 and 100 nM $1,25(OH)_2D_3$ and assessed the cytokine levels in the supernatant. Using a concentration of 10 and 100 nM $1,25(OH)_2D_3$, tumor necrosis factor (TNF)α and IL-6 production was decreased by more than 50–85%, in a

dose-dependent manner ($P < 0.05$). Reduction in interferon (IFN)γ secretion was apparent at even the lowest dose of 1 nM $1,25(OH)_2D_3$ and it was significantly suppressed with a greater than 90% drop at 100 nM $1,25(OH)_2D_3$. On the contrary, IL-10 production was increased by 1.5-2.5 folds at 10 and 100 nM, respectively (Figure 1). These results implied that

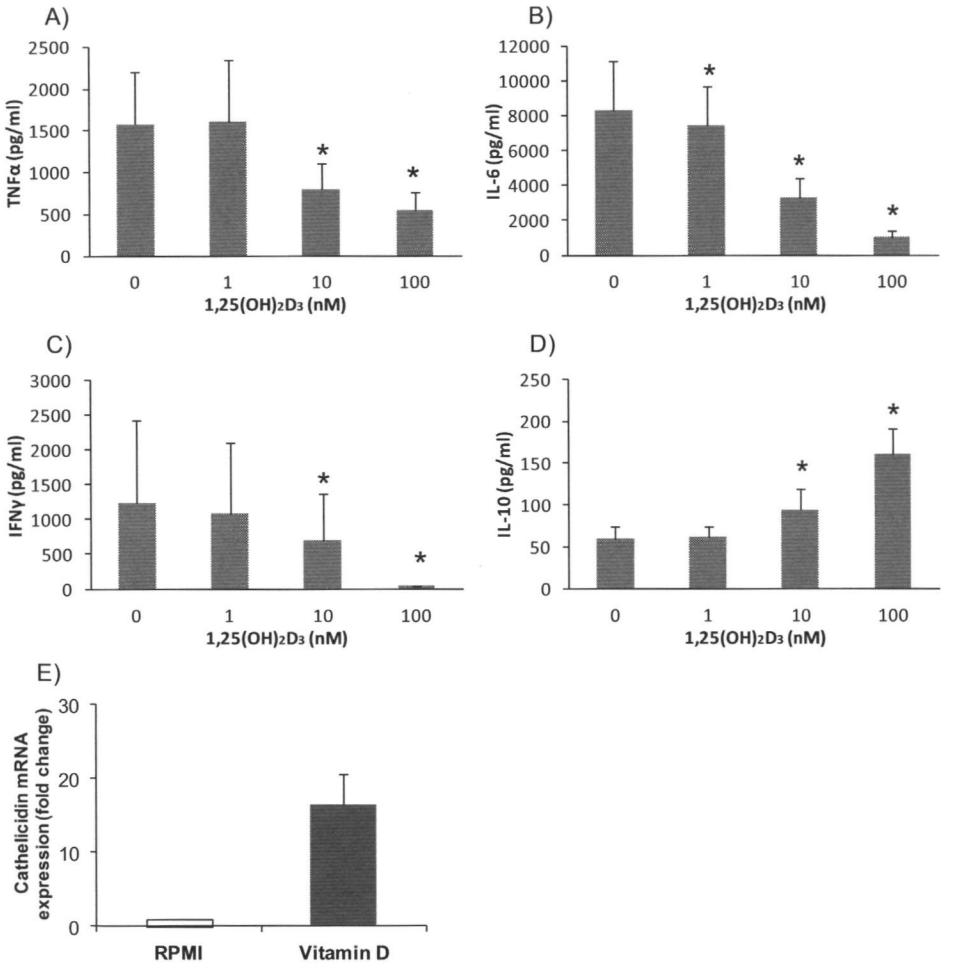

Figure 1. $1,25(OH)_2D_3$ modulates cytokine response and antimicrobial peptide production to MTB

PBMC were incubated with 1-100 nM $1,25(OH)_2D_3$ and stimulated with 10^5 HK MTB. Supernatants were collected and analyzed for: (A) TNFα, (B) IL-6, (C) IFNγ and (D) IL-10 at 24 h. (E) $1,25(OH)_2D_3$ increases the expression of the cathelicidin at the transcription level at 24 h. PBMC were incubated with and without 100 nM $1,25(OH)_2D_3$ and stimulated with 10^5 HK MTB. Quantitative PCR for human cathelicidin gene was normalized to β2M gene expression. Data show results from six independent experiments performed with cells obtained from different donors. $*P < 0.05$ as compared to respective cell culture without the addition of $1,25(OH)_2D_3$.

$1,25(OH)_2D_3$ is able to modify the cytokine response to *M. tuberculosis* towards an anti-inflammatory profile.

$1,25(OH)_2D_3$ induces the production of cathelicidin

We also examined the antimicrobial effect of vitamin D_3 by measuring cathelicidin production, which is an important component of anti-mycobacterial host defense. Despite the attenuated proinflammatory cytokine profile, the transcription of the antimicrobial peptide cathelicidin was up-regulated 16-fold by adding 100 nM $1,25(OH)_2D_3$ to the system (Figure 1E). Henceforth, $1,25(OH)_2D_3$ is able to induce antimicrobial defence in response to M. tuberculosis.

$1,25(OH)_2D_3$ does not attenuate cytokine response to *M. tuberculosis* through induction of SOCS or modulation of T cell differentiation

From the above observations, we postulated that $1,25(OH)_2D_3$ could have modulated the proinflammatory cytokine response to *M. tuberculosis* via induction of the suppressor of cytokine signaling (SOCS) proteins or modulation of T cell differentiation pathways. We hypothesized that $1,25(OH)_2D_3$ could skew T cell differentiation towards a Th2 or regulatory

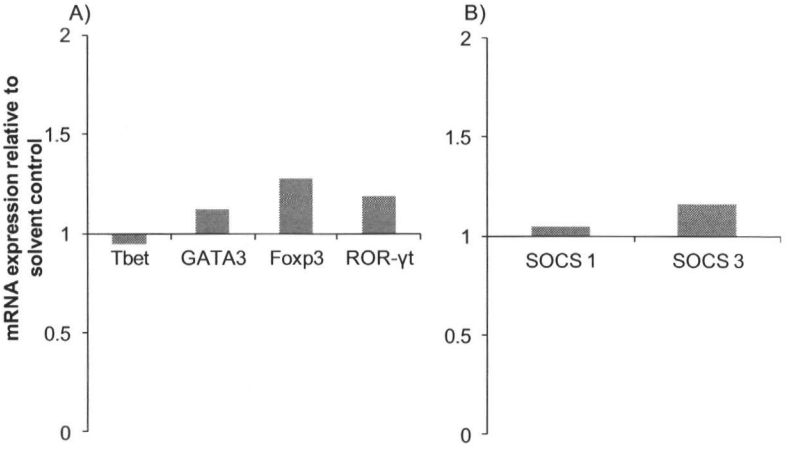

Figure 2. Vitamin D_3 does not modulate T helper differentiation or SOCS expression

(A) $1,25(OH)_2D_3$ does not affect T cell transcription factors. (B) There is no significant effect by $1,25(OH)_2D_3$ on SOCS1 and SOCS3. Quantitative PCR for human Tbet, GATA3, Foxp3, ROR-γt, SOCS1 and SOCS3 was conducted with PBMC and normalized to β2M gene expression. Cells were stimulated with 10^5 HK MTB and incubated with 10 nM $1,25(OH)_2D_3$ or solvent for 24 or 4 h (SOCS1 and 3). Data show results from five independent experiments performed with cells obtained from different donors and are expressed as fold change relative to controls.

T cell (Treg) phenotype. Therefore, we performed quantitative polymerase chain reaction (PCR) to analyze the gene expression of the various T cell transcription factors upon *M. tuberculosis* stimulation on PBMC, in the presence of $1,25(OH)_2D_3$. We found no significant changes in T-bet, GATA3, Foxp3 or ROR-γt mRNA expression (Figure 2A) 24 h after stimulation. We also assessed SOCS1 and SOCS3 mRNA expression under the same conditions and found no significant effects induced by $1,25(OH)_2D_3$ (Figure 2B).

TLR2 and TLR4 mRNA and protein expression were decreased by $1,25(OH)_2D_3$

TLR2, TLR4, Dectin-1 and mannose receptors (MR) are pattern recognition receptors (PRR) known to be involved in *M. tuberculosis* infection. Hence, we investigated whether $1,25(OH)_2D_3$ could have acted through the various PRRs. We stimulated PBMC with *M. tuberculosis*, with or without 100 nM $1,25(OH)_2D_3$, or with 100 nM $1,25(OH)_2D_3$ followed by 1000 nM vitamin D receptor (VDR) antagonist. Flow cytometric analysis of the various receptors was performed at 24, 48 h and day 4, and quantitative PCR was carried out to analyze the gene expression of TLR2 and TLR4 at 24 h. The surface expression of both TLR2 and TLR4 was reduced by $1,25(OH)_2D_3$ (Figure 3A and B) at 24, 48 h and day 4; data not shown for 24 h and day 4. Furthermore, TLR2 and TLR4 mRNA was decreased by $1,25(OH)_2D_3$ at 24 h and was restored by the addition of a VDR antagonist, ZK159222 (Figure 3C).

Reduced C-type lectin receptors expression mediated by $1,25(OH)_2D_3$ could be reversed by a VDR antagonist

With the same experimental set-up as above, we analyzed the effects of $1,25(OH)_2D_3$ on C-type lectin receptors (CLR), namely Dectin-1 and mannose receptors. The surface expression of Dectin-1 receptor and MR was also inhibited by $1,25(OH)_2D_3$ (Figure 4A and B) at 48 h and day 4 of incubation with *M. tuberculosis*, respectively. The expression of Dectin-1 receptor on flow cytometry was also reduced at 24 h and day 4 (data not shown), but MR expression was not apparent until day 4. The mRNA levels of these two receptors were also reduced in the presence of $1,25(OH)_2D_3$ at 24 h. This effect could be reversed by the VDR antagonist ZK159222 (Figure 4C).

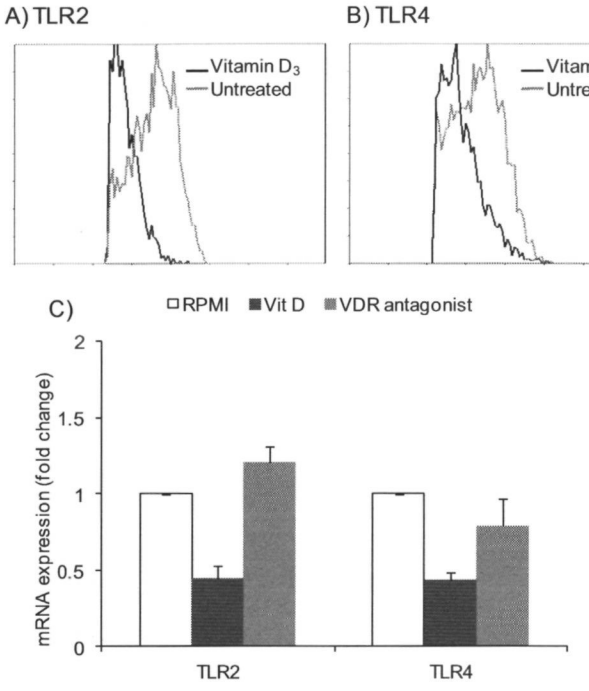

Figure 3. $1,25(OH)_2D_3$ inhibits TLR2 and TLR4 expression

Representative histogram overlay plots show decreased: (A) TLR2 and (B) TLR4 expression at 48 h with $1,25(OH)_2D_3$ treatment as assessed by flow cytometry. (C) $1,25(OH)_2D_3$ down-modulates the expression of these PRRs at the transcription level at 24 h and VDR antagonist reverse this effect. PBMC were incubated with and without 100 nM $1,25(OH)_2D_3$ or with 100 nM $1,25(OH)_2D_3$ followed by 1000 nM VDR antagonist, ZK159222; and stimulated with 10^5 HK MTB. Quantitative PCR for human TLR2 and TLR4 were normalized to β2M gene expression. Data show results from six independent experiments performed with cells obtained from different donors and are expressed as fold change relative to controls.

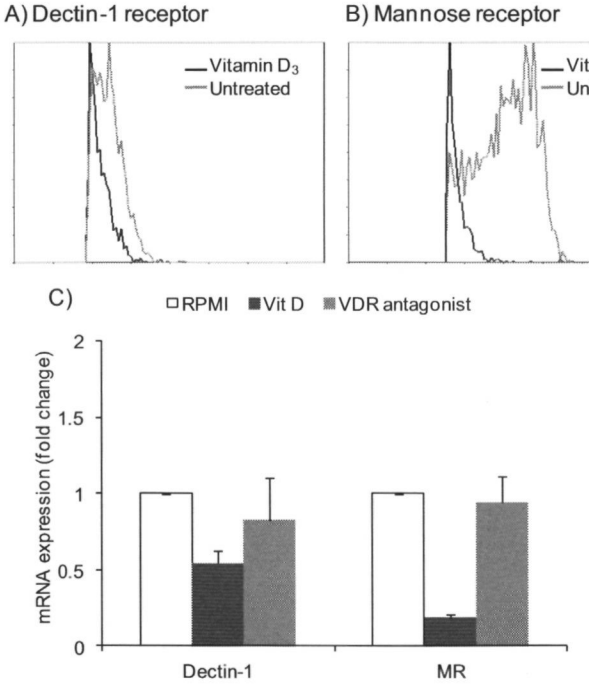

Figure 4. $1,25(OH)_2D_3$ suppress Dectin-1 receptor and MR expression

Representative histogram overlay plots show $1,25(OH)_2D_3$ down-regulate the expression of: (A) Dectin-1 receptor and (B) MR with $1,25(OH)_2D_3$ treatment as assessed by flow cytometry. (C) $1,25(OH)_2D_3$ suppresses the expression of Dectin-1 receptor and MR at the transcription level at 24 h. Treatment with VDR antagonist reverses this effect. PBMC were incubated with and without 100 nM $1,25(OH)_2D_3$ or with 100 nM $1,25(OH)_2D_3$ followed by 1000 nM VDR antagonist, ZK159222; and stimulated with 10^5 HK MTB. Quantitative PCR for human Dectin-1 and MR were normalized to β2M gene expression. Data show results from six independent experiments performed with cells obtained from different donors and are expressed as fold change relative to controls.

DISCUSSION

Although the use of pharmacological doses of vitamin D_3 in the treatment of tuberculosis is no longer in practice due to the availability of anti-mycobacterial chemotherapy, the immunomodulatory role of vitamin D_3 in TB has gained an increased research interest. This study demonstrates that $1,25(OH)_2D_3$ down-modulate host proinflammatory cytokine response to *M. tuberculosis,* while inducing the antimicrobial peptide cathelicidin. This effect on cytokine profile is mediated by VDR-dependent modulation of the various key PRRs involved in *M. tuberculosis,* TLR2, TLR4 and the C-type lectin receptors-Dectin-1 and MR.

The most important observation of the present study is that while vitamin D_3 down-modulates the proinflammatory cytokine profile, it induces an antimicrobial response. We demonstrated a 16-fold increase in the antimicrobial peptide cathelicidin production with vitamin D_3 treatment upon *M. tuberculosis* challenge. As reported previously, induction of cathelicidin by vitamin D_3 is largely accountable for its antimicrobial activity against intracellular *M. tuberculosis* (23). Here, we also showed that $1,25(OH)_2D_3$ differentially influenced cytokine profile during *M. tuberculosis* challenge, with production of the proinflammatory cytokines IFNγ, TNFα and IL-6 notably reduced, while production of the anti-inflammatory cytokine IL-10 was up-regulated. In an *ex vivo* study using slightly different stimulation with *M. tuberculosis* antigen and live *M. tuberculosis,* Vidyarani et al described decreased IL-6, IL-8, IL-10, IL-12p40 and IFNγ and increased IL-4 production by $1,25(OH)_2D_3$ (24). Here, we went a step beyond to evaluate the mechanism of action through which $1,25(OH)_2D_3$ modulates cytokine production. It has been previously shown that vitamin D_3 can modify the balance between Th1 and Th2 response (25-27), and we hypothesized that the increased IL-10 seen in our TB model could be the result of skewing towards Th2 differentiation pathway or the expansion of Treg population (28;29). However, there were no notable shifts in the T helper cell or Treg transcription factors: T-bet, GATA3, Foxp3 or ROR-γt mRNA expression was not changed by $1,25(OH)_2D_3$ treatment in our study. Since there was significant suppression of IL-6 and IFNγ by $1,25(OH)_2D_3$, we investigated whether the underlying mechanism was due to unknown $1,25(OH)_2D_3$ effects on the SOCS3 and SOCS1 regulatory pathways (30). However, there was no pronounced stimulation of SOCS1 and SOCS3 mRNA by $1,25(OH)_2D_3$ that could have been related with the down-regulation of proinflammatory cytokine production.

We next investigated whether alteration of PRR expression could account for the shift in the cytokine balance induced by $1,25(OH)_2D_3$. Interestingly, $1,25(OH)_2D_3$ reduced surface expression of TLR2 and TLR4 and when investigated further revealed that its action was attributable to upstream inhibition at the mRNA transcription level. Upon reversal of inhibition by a VDR antagonist, we validated that this effect was mediated by VDR signaling. The detection of pathogen-associated molecular patterns by PRRs is crucial for the initiation and coordination of the immune response against mycobacterial infections. TLR signaling is the main arm of innate immunity upon challenged by *M. tuberculosis,* with involvement of primarily TLR2, TLR4 and TLR9 (31;32). Previously, Liu et al reported that activation of TLR2/TLR1 on human macrophages increased $1,25(OH)_2D_3$ synthesis by these cells. This consequently induced an up-regulation of the antimicrobial peptide cathelicidin, leading to killing of intracellular *M. tuberculosis* (20). Here, we showed a converse relationship with $1,25(OH)_2D_3$ acting via VDR to down-regulate TLR2 expression. This finding brings about a new perspective how VDR and TLR signaling pathways interact. Another interesting point is the observation of a down-modulation of Th1 cytokine response in parallel with TLR2 expression. This may suggest that TLR2 signaling negatively modulates macrophage functions and curtails Th1 activation (33;34).

Besides the TLRs, Dectin-1 and MR are also related to macrophages' activity against *M. tuberculosis* (35-37). Of note, we were able to demonstrate that $1,25(OH)_2D_3$ can also down-regulate Dectin-1 and MR surface and mRNA expression. To date, only the TLRs have been reported to be associated with the immunologic activities of vitamin D_3 and VDR signaling pathway. This is the first study to report effects of vitamin D_3 on the expression of C-type lectin receptors. In addition, our results also suggest that the immunomodulatory effects of $1,25(OH)_2D_3$ against *M. tuberculosis* arise from its influence on innate, rather than acquired immune response. This interpretation is in keeping with an *in vitro* study conducted to evaluate innate and acquired host response in TB contacts. While a single oral dose of 2.5 mg ergocalciferol led to an increase in serum and supernatant $25(OH)D_3$ concentration, the effect seemed to be primarily enhanced innate immunity to mycobacterial infection (15).

Vitamin D_3 has no direct anti-mycobacterial action and several modes of action have been put forward. Earlier work in the 1980s revealed that $1,25(OH)_2D_3$ improve the bactericidal activity against *M. tuberculosis in vitro* in both monocytes (38) and macrophages (39). Others have shown that $1,25(OH)_2D_3$ generates a superoxide burst (40) and enhances phagosome–lysosome fusion in macrophages infected with *M. tuberculosis* (41). Our

findings on $1,25(OH)_2D_3$ dampening the proinflammatory cytokine response through TLR2/4, Dectin-1 and MR signaling provided new insights on the immunologic capacity of vitamin D_3 towards M. tuberculosis.

Contrary to the common belief that Th1 cytokines are beneficial in TB treatment outcome (42;43), our data showed that vitamin D_3 skew the cytokine balance away from a Th1 phenotype. The signature cytokine of Th1 responses, IFNγ, contributes to protective immunity against *M. tuberculosis* (44), while TNFα plays an important role in activating macrophages and facilitating granuloma formation (45). However, excessive secretion and release of TNFα into the circulation generate systemic side effects such as fever and wasting as well as local deleterious effects in the tissues, which can account for clinical deterioration early in treatment (46). Interleukin-6 has both pro- and anti-inflammatory properties and it is said to be harmful in mycobacterial infections due to its inhibitory effects on IFNγ and TNFα (47), as well as its ability to promote *in vitro* growth of *Mycobacterium avium* (48).

Interleukin-10 is an anti-inflammatory cytokine and has been associated with depressed T cell IFNγ response against *M. tuberculosis* (49;50). Given that Th1 cytokines are often found along with IL-10, we suppose it is the relative balance between these two cytokines that determines whether the protective immunity against *M. tuberculosis* is suppressed. One study noted significantly higher levels of TNFα, IFNγ, IL-10 and IL-12p40 in active pulmonary TB cases as compared to inactive cases and healthy controls. With anti-mycobacterial therapy, TNFα, IFNγ and IL-10 concentrations were decreased (51). In another study, TNFα production was elevated while IFNγ secretion was reduced in active pulmonary TB patients as compared to healthy individuals. IFNγ trends correlated inversely with disease severity and were reversed with increase in IFNγ/IL-10 ratios during anti-mycobacterial therapy (43). In our study, we showed that vitamin D_3 is capable of inducing the production of antimicrobial peptide cathelicidin, while limiting the proinflammatory cytokine response. An unrestrained proinflammatory response may give rise to excessive tissue damage. On the other hand, anti-inflammatory cytokines limit tissue injury by inhibiting excessive inflammatory response. Therefore, regulating the pro- and anti-inflammatory cytokine response influences the outcome of tuberculosis treatment We speculate from our data that by manipulating vitamin D_3 therapy to attain an anti-mycobacterial effect while limiting excessive proinflammatory response during TB, desirable treatment outcome could be achieved. Having said that, future studies are warranted to optimise this model and to

identify the vitamin D dosages that may exert beneficial effects during disease. In addition, future animal studies to strengthen and validate the above findings and the potential value of adjunctive vitamin D_3 therapy in TB are warranted, as well as to find the optimal dosage regimen to attain the desired therapeutic effects. Further studies on the interplay between TNFα and IFNγ with cathelicidin in an *in vivo* system may elucidate key information on achieving protective immunity, while limiting excessive collateral tissue damage implied by our *in vitro* model.

In conclusion, we describe immunomodulatory effects of $1,25(OH)_2D_3$ that dampens proinflammatory cytokine responses as a result of inhibition of TLR and CLR expression. On the other hand, $1,25(OH)_2D_3$ induces antimicrobial effects by up-regulating the production of cathelicidin. Future studies are warranted to investigate the role of vitamin D_3 in controlling cytokine dynamics in different tuberculosis patient cohorts, to gain greater knowledge on how we can best employ this therapy to augment the outcome and prognosis of TB.

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1,25-dihydroxyvitamin D_3 Modulates Cytokine Production Induced by *Candida albicans*: Impact of Seasonal Variation of Immune Responses

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ABSTRACT

Our interest in the immunological effects produced by vitamin D_3 ($1,25(OH)_2D_3$) and its therapeutic potential prompted us to examine the role of $1,25(OH)_2D_3$ on cytokine production induced by *Candida albicans.* Peripheral blood mononuclear cells (PBMC) were stimulated with *C. albicans* and $1,25(OH)_2D_3$, cytokine concentrations were measured in culture supernatant. Quantitative polymerase chain reaction (qPCR) was performed for T cell transcription factors, suppressor of cytokine signaling (SOCS)1 and 3. Toll-like receptor (TLR)2/4, Dectin-1, and mannose receptor (MR) expression was studied using flow cytometry and qPCR. An *ex vivo* stimulation study was carried out in healthy volunteers to investigate the seasonality of immune response to *C. albicans*. Upon *in vitro C. albicans* stimulation, $1,25(OH)_2D_3$ induced a dose-dependent, down-regulation of IL-6, TNFα, IL-17, and IFNγ. It also increased IL-10 production. The shift in cytokine profile was not due to $1,25(OH)_2D_3$ augmenting expression of either T helper differentiation factors or SOCS1 and SOCS3 mRNA. $1,25(OH)_2D_3$ inhibited TLR2, TLR4, Dectin-1, and MR mRNA and protein expression. In our seasonality study, both IL-17 and IFNγ levels were suppressed in summer when $25(OH)D_3$ levels were elevated. Vitamin D_3 skews cytokine responses toward an anti-inflammatory profile, mediated by suppression of TLR2, TLR4, Dectin-1, and MR transcription, leading to reduced surface expression. The biological relevance of these effects has been confirmed by the seasonality of cytokine responses.

INTRODUCTION

Beyond its classical role on bone metabolism, vitamin D_3 has now been widely accepted as a potent modulator of immune function affecting various cell types. This view is based on several fundamental observations (1). First, vitamin D receptor (VDR), through which biologically active $1,25(OH)_2D_3$ acts, is expressed by most immune cells, including T lymphocytes, neutrophils, macrophages, and dendritic cells (1-4). Second, T lymphocytes, macrophages, and dendritic cells are equipped with enzymes to metabolize $25(OH)D_3$ into $1,25(OH)_2D_3$ (5-7). Third, $1,25(OH)_2D_3$ can influence both innate and adaptive immune cell types. Vitamin D_3 has been shown to increase the proliferation and maturation of monocytes to macrophages *in vitro* (8;9). It also suppresses T cell proliferation and differentiation (10). $1,25(OH)_2D_3$ is known to induce a more tolerogenic phenotype in dendritic cells (11). In addition, it can skew T cell response toward a T helper (Th) 2 profile (7;12).

The above immunomodulatory properties of vitamin D_3 have fueled much interest on vitamin D_3 potential as a therapeutic agent in infectious diseases. Most notably, the clinical course of tuberculosis (TB) (13), as well as viral influenza and human immunodeficiency virus (HIV) infection, has been associated with vitamin D_3 status (14;15). However, the capability of vitamin D_3 to modulate the host immune response to fungal infections is still unknown. Invasive candidiasis remains a disease with significant mortality and morbidity in hospitalized patients (16). Both innate and adaptive immunity are essential for optimal defence against fungal infections. Despite the current range of available antifungal therapy, mortality rates from invasive candidiasis remains high. This is partly due to the host's inability to mount a successful immune response to the invading fungi.

We know that vitamin D_3 is a powerful immunomodulator. However we do not know its potential impact on *Candida albicans* infection. We hypothesize that $1,25(OH)_2D_3$ can modulate the innate immune response of human leukocytes challenged with *C. albicans*. We therefore surmise that the knowledge gained in this study will increase our understanding of the potential of vitamin D_3 as an adjunct immunotherapeutic agent against invasive fungal infections. In addition, due to the seasonal human variability of vitamin D_3 stores, we hoped to further validate our *in vitro* findings in a non-interventional, *ex vivo* setting by measuring cytokine response to *C. albicans* stimulation in a small cohort of healthy volunteers over a 1-year period.

MATERIALS AND METHODS

Micro-organisms

Heat-killed *C. albicans* blastoconidia, a strain of American Type Culture Collection MYA-3573 (UC820), were used at a concentration of 10^5 micro-organisms/mL as previously described (17;18).

Reagents

Toll-like receptor (TLR)2 ligand lipopeptide (S)-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys4-OH, trihydrochloride (Pam3Cys), was purchased from EMC Microcollections (Tübingen, Germany). TLR4 ligand lipopolysaccharide (LPS; E. coli serotype 055:B5) was purchased from Sigma Chemical Co (St Louis, MO, USA). An extra purification step of LPS was performed as previously described (19). Particulate β-glucan was a kind gift from Dr. David Williams (University of Tennessee) (20). $1,25(OH)_2D_3$ was purchased from Fluka Biochemika, Sigma-Aldrich (Missouri, USA) and dissolved in absolute ethanol.

Stimulation assays

Venous blood was drawn into EDTA tubes from healthy volunteers after informed consent. The study protocol was approved by institutional ethics committee. PBMC were isolated by density centrifugation on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). Cells were washed twice in saline and counted. The cell counts were adjusted to $5x10^6$ cells/mL. A 100 µL volume of PBMC, suspended in culture medium (RPMI 1640 DM; ICN Biomedicals, Costa Mesa, CA) and supplemented with 10 µg/mL gentamicin, 10 mM L-glutamine, 10 mM pyruvate, and 10% human pooled serum was added to flat-bottomed, 96-well plates (Greiner, The Netherlands).

PBMC were preincubated with $1,25(OH)_2D_3$ (at the respective doses indicated) for 30 minutes, followed by addition of *C. albicans* at 10^5 micro-organisms/mL or RPMI (as unprimed control). This concentration of *C. albicans* has been predetermined in pilot experiments to yield optimal cytokine response. In the stimulation experiments using specific ligands, PBMC were incubated with the various stimuli individually (or in combination): TLR2 ligand (Pam3Cys 10 µg/mL), TLR4 ligand (LPS 1 ng/mL), Dectin-1 ligand (β-glucan 20 µg/mL), or combinations of β-glucan/Pam3Cys and β-glucan/LPS. Cell cultures were incubated in a 37°C, 95% humidity, 5% CO_2 incubator. The culture supernatants were collected after 24 or 48 hours or 7 days of incubation, as appropriate, and stored at -20°C until cytokine assay.

Flow cytometry

Cells were phenotypically analyzed by 5-color flow cytometry (Coulter Cytomics FC 500, Beckman Coulter, Fullerton, USA) using Coulter Epics Expo 32 software. Cells were washed with PBS with 0.2% bovine serum albumin (BSA) before being labeled with fluorochrome-conjugated antibodies (mAb). After incubation for 20 minutes in the dark at room temperature, cells were washed twice to remove unbound antibodies and analyzed. For cell-surface staining, the following mAb were used: Dectin-1 receptor-PE (259931, R&D Systems, Minneapolis, MN), along with TLR2-FITC (TL2.1) and TLR4-PE (HTA125) (both from eBioscience, San Diego, CA) and mannose receptor (MR)-FITC (19.2, BD Bioscience, New York, NY).

Cytokine measurements

Interleukins (IL)-6, IL-1β, IL-10 and interferon (IFN)γ concentrations were measured by commercial sandwich ELISA kits (Pelikine Compact, CLB, Amsterdam, The Netherlands) according to manufacturer instructions. Human tumor necrosis factor alpha (TNFα) and IL-17 were measured by the appropriate commercial ELISA kits (R&D Systems, Minneapolis, MN). Detection limits were 8 pg/mL (IL-6 and IL-10), 20 pg/mL (IL-1β and IFNγ), and 40 pg/mL (TNF-α and IL-17).

Quantitative polymerase chain reaction (qPCR)

To determine mRNA expression, we extracted RNA from PBMC stimulated with *C. albicans* in both the presence and absence of $1,25(OH)_2D_3$ for 24 hours. We extracted that RNA from 107 PBMC by using 1 mL TRIzol reagent (Sigma, St. Louis, MO). Subsequently, 200 µL chloroform and 500 µL 2-propanol (Merck, Darmstadt, Germany) were used to separate the RNA from DNA and proteins. Finally, after washing with 75% ethanol, we dissolved the dry RNA in 50 µL of diethylpyrocarbonate (DEPC) water. The amount and quality of mRNA were determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Complementary DNA was synthesized with a Superscript Reverse Transcriptase (Invitrogen).

Quantitative, real-time PCR was performed using the Bio-Rad iCycler and SYBR Green. The following primers were used (5'-3'):

1.AACCTCAGACAAAGCGTCAAATC (forward) and ACCAAGATCCAGAAGAGCCAAA (reverse) for TLR2, TTCCTTCAACCAAGAACATAGATC (forward) and TTGTTTCAATTTCACACCTGGATAA (reverse)

2.for TLR4, ACAATGCTGGCAACTGGGCT (forward) and CCGAGAAAGGCCTATCCAAAA (reverse) for Dectin-1 receptor, TCAAGACAATCCACCAGTTACT (forward) and TTCTCTTTGCTGAAATAATACTGGTAGTC (reverse)
3.for MR, GCAGCCGACAATGCAGTCT (forward) and GAACGGAATGTGCGGAAGTG (reverse) for SOCS1, TGCGCCTCAAGACCTTCAG (forward) and GAGCTGTCGCGGATCAGAAA (reverse) for SOCS3 and
4.ATGAGTATGCCTGCCGTGTG (forward) and CCAAATGCGGCATCTTCAAAC (reverse) for β2 microglobulin (B2M) (Biolegio, The Netherlands) as housekeeping gene.

In another system, we quantified transcripts by real-time quantitative PCR on an ABIPRISM 7700 Sequence Detector using predesigned TaqMan GeneExpression Assays and reagents according to manufacturer instructions (Applied Biosystems, Foster City, CA). Probes with the following Applied Biosystems assay identification numbers were used: TBX21 (Tbet), Hs00203436_mL; GATA3, Hs00231122_mL; FOXP3, Hs00203958_mL; RORC1-2, Hs00172858_mL; RORC10-11, Hs01076112_mL, using human HPRT1 Endogenous Control (4333768T; Applied Biosystems) acted as housekeeping gene.
We validated all primers according to protocol. Mean relative mRNA expression was calculated using Pfaffl method. Values are expressed as ratio of fold increase to mRNA levels of vitamin D_3-untreated cells.

***Ex vivo* study**

We recruited 15 healthy male volunteers from Radboud University Nijmegen Medical Centre, The Netherlands. Venous blood was drawn from all subjects on 4 occasions—in winter, spring, summer, and autumn of 2009. PBMC were isolated and stimulation assay was performed with *C. albicans* as discussed. This study has been approved by the local ethics committee. Informed consent was obtained from all volunteers.

Vitamin D_3 measurement

Serum 25(OH)D_3 levels were determined using high-performance liquid chromatography (HPLC) with UV detection, after prior extraction on small SepPak columns. Tritiated 25(OH)D_3, collected from the HPLCsystem during passage of the UV peak, corrected for procedural losses.

Statistical analysis

Results from at least 5 experiments were pooled and analyzed using SPSS 16.0 statistical software. Data given as means $\pm$ SEM and the Wilcoxon signed rank test compared differences between groups. Significance level was set at $P < 0.05$.

RESULTS

$1,25(OH)_2D_3$ down-modulate proinflammatory cytokine response to *C. albicans*

The influence of $1,25(OH)_2D_3$ on immune response to *C. albicans* was assessed by stimulating PBMC in the presence of 1, 10, and 100 nmol/L of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ down-regulated TNFα secretion (25% drop) as well as IL-6 and IL-17 production (by more than 70%, in a dose-dependent manner ($P < 0.05$). IFNγ secretion was also attenuated by $1,25(OH)_2D_3$. In contrast, IL-10 production is increased 1.5-fold (although this was statistically insignificant) (Figure 1). These observations suggested that $1,25(OH)_2D_3$ has the capacity to modulate host inflammatory response to *C. albicans* toward an anti-inflammatory response.

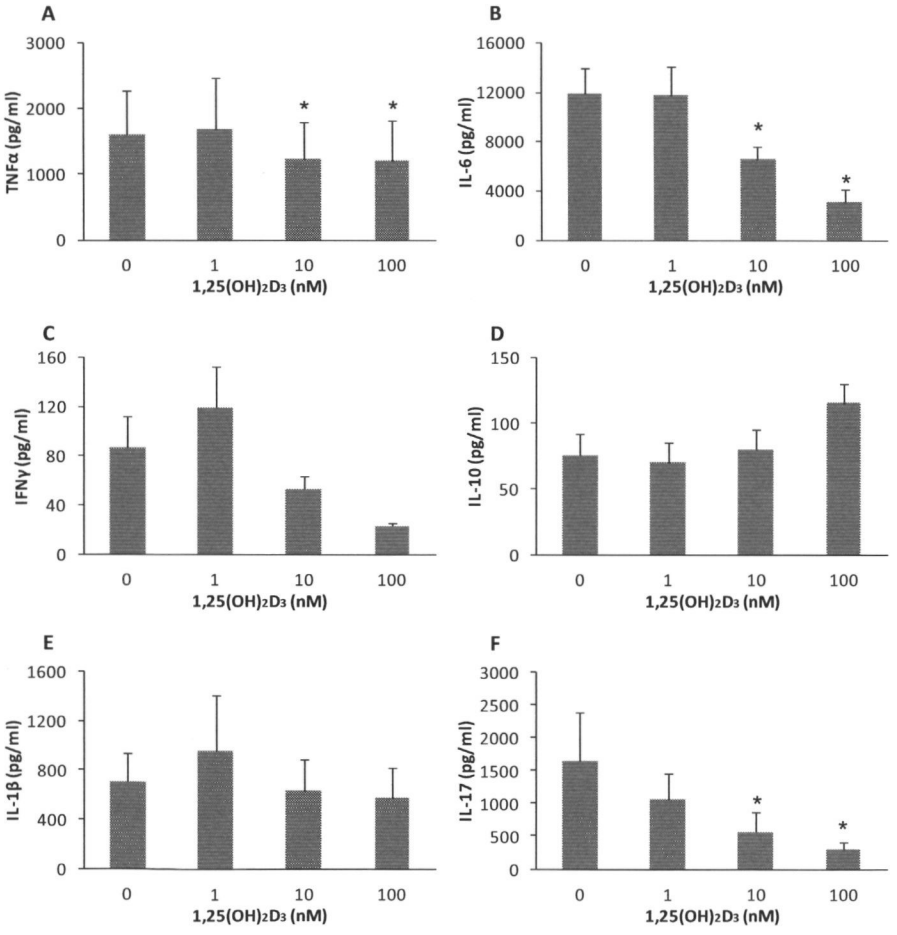

Figure 1. $1,25(OH)_2D_3$ attenuates the cytokine response to *Candida albicans*

PBMC were incubated in the absence or presence of various $1,25(OH)_2D_3$ concentrations ranging from 1 to 100 nmol/L and stimulated with 10^5 HK *C. albicans*. Supernatants were collected and

analyzed for A) TNFα, B) IL-6, C) IFNγ, D) IL-10 and E) IL-1β at 24 h and F) IL-17 at day 7. Data show results from 6 independent experiments performed with cells obtained from different donors. *P < 0.05 as compared to respective cell culture without the addition of $1,25(OH)_2D_3$.

Modulatory effects of $1,25(OH)_2D_3$ are not mediated through SOCS or skewing of T cell differentiation

Two possible mechanisms through which $1,25(OH)_2D_3$ could have attenuated proinflammatory response to *C. albicans* was through disruption of the suppressors of cytokine signaling (SOCS) pathway or a shift in T cell differentiation away from Th1 and Th17 toward Th2 or regulatory T cell (Treg) profile. To assess the gene expression profiles of the various T cell transcription factors in response to $1,25(OH)_2D_3$, we performed quantitative PCR after stimulating PBMC with *C. albicans.* There were no significant changes in Tbet, GATA3, Foxp3, or ROR-γt mRNA expression (Figure 2A) 24 hours after cells were stimulated with *C. albicans* in the presence of $1,25(OH)_2D_3$. We examined the effect of $1,25(OH)_2D_3$ on SOCS1 and SOCS3 mRNA expression under the same conditions and found a moderate drop in SOCS1 at 4 hours (Figure 2B), but this cannot explain a decrease in the induction of proinflammatory cytokines.

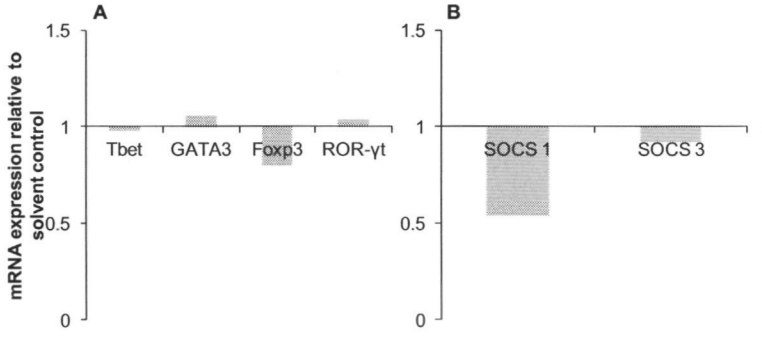

Figure 2. $1,25(OH)_2D_3$ does not act through modulation of T helper differentiation or SOCS pathway

A) $1,25(OH)_2D_3$ does not modulate of T cell transcription factors. B) Modulation of SOCS1 and SOCS3 mRNA expression by $1,25(OH)_2D_3$. Quantitative PCR for human Tbet, GATA3, Foxp3, ROR-γt, SOCS, and SOCS3 was conducted with PBMC and normalized to HPRT1 or β2M (for SOCS1 and 3) gene expression. Cells were incubated with 10 nmol/L $1,25(OH)_2D_3$ or solvent for 24 h or 4 h (SOCS1 and 3). Data show results from 5 independent experiments and are expressed as fold change relative to solvent control.

Reduced TLR2 and TLR4 mRNA and protein expression by $1,25(OH)_2D_3$

TLR2, TLR4, Dectin-1 and MR are well-established pattern recognition receptors (PRR) for the detection of *C. albicans*. Therefore, we investigated whether $1,25(OH)_2D_3$ could also modulate the expression of PRRs. In PBMC incubated with *C. albicans*, the expressions of both TLR2 and TLR4 were reduced by $1,25(OH)_2D_3$ at 24 hours, 48 hours (Figure 3A-3D), and day 4 (not shown). In addition, TLR2 mRNA was decreased by $1,25(OH)_2D_3$ after 24 hours incubation, while a less remarkable effect was apparent on the TLR4 expression (Figure 3G).

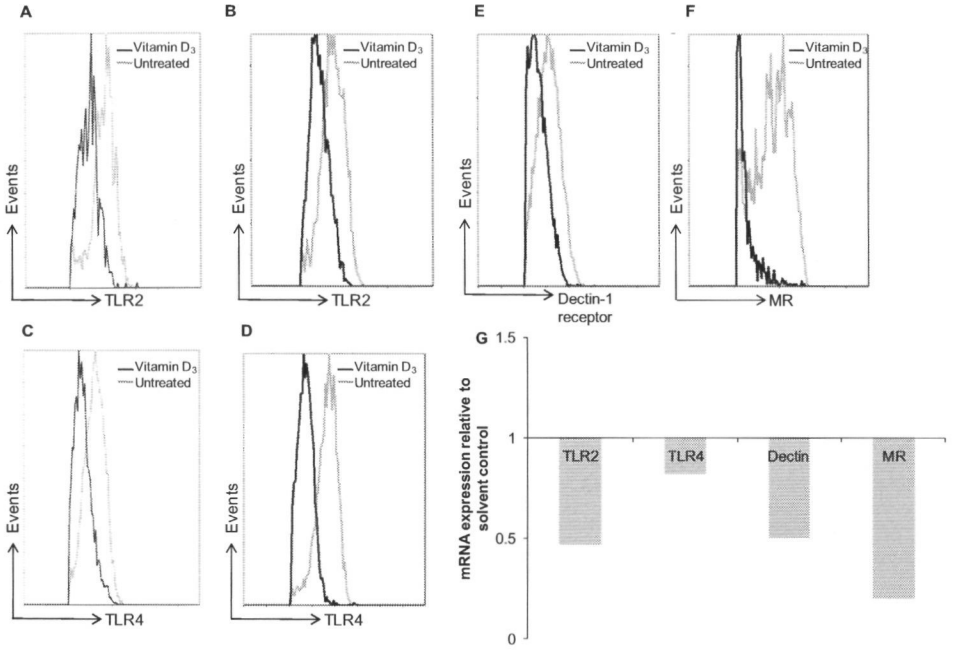

Figure 3. $1,25(OH)_2D_3$ inhibits expression of TLR2, TLR4, Dectin-1 receptor and MR

Representative histogram overlay plots show reduced A and B) TLR2 (at 24 h and 48 h), C and D) TLR4 (at 24 h and 48 h), E) Dectin-1 (at 48 h) and F) MR (day 4) with and without $1,25(OH)_2D_3$ treatment as assessed by flow cytometry. G) $1,25(OH)_2D_3$ down-modulates TLR2, TLR4, Dectin-1 and MR mRNA expression. PBMC were incubated in the absence or presence of 100 nmol/L $1,25(OH)_2D_3$ and stimulated with 10^5 HK *Candida albicans* . Flow cytometry data shown was performed at 24 h and 48 h for TLR2, TLR4; 48 h for Dectin-1; and day 4 for MR. Quantitative PCR for human TLR2, TLR4, Dectin-1, and MR (normalized to β2M gene expression) was performed at 24 h. Data represent 5 independent experiments performed with cells obtained from different donors.

$1,25(OH)_2D_3$ diminished Dectin-1 and mannose receptors expression at the level of transcription

The surface expression of Dectin-1 was inhibited by $1,25(OH)_2D_3$ upon stimulation with *C. albicans*, and this was most evident at 48 hours (Figure 3E); data at 24 hours and day 4 is

not shown. Down-regulation of MR surface expression by $1,25(OH)_2D_3$ is clearly seen by day 4 (Figure 3F) even though MR protein expression on PBMC is usually only apparent after 72 hours of incubation. Likewise, the mRNA levels of both receptors were also reduced in the vitamin D_3-treated cells (Figure 3G).

Functional consequence of $1,25(OH)_2D_3$-induced suppression of TLRs and C-Type lectin receptor expression

To validate our findings and to assess the functional consequences of vitamin D_3 on TLRs and C-type lectin receptor (CLR) expression, we carried out stimulation using purified TLR2, TLR4, and Dectin-1 ligands in PBMC in the presence of $1,25(OH)_2D_3$. A significant drop in IL-6 and TNFα secretion was observed in cells treated with $1,25(OH)_2D_3$. In addition, we used β-glucan combined with Pam3Cys or LPS to test the effect of $1,25(OH)_2D_3$ on synergy

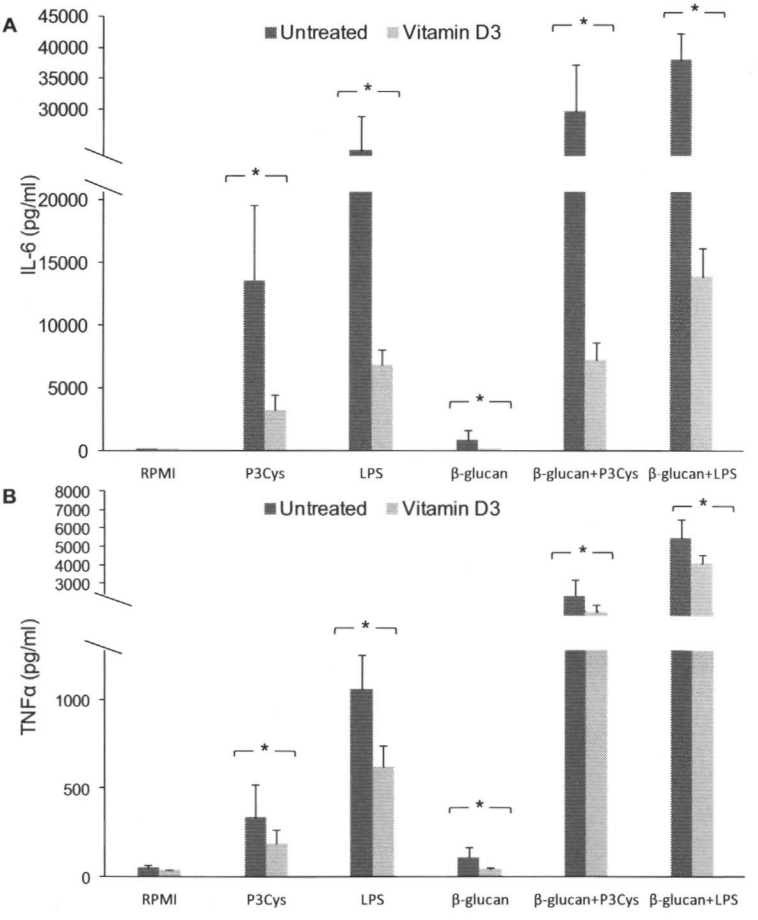

Figure 4. $1,25(OH)_2D_3$ inhibited proinflammatory cytokines induced by purified TLR/Dectin-1 ligands

PBMC were incubated in the presence or absence of 100 nmol/L $1,25(OH)_2D_3$ and stimulated with 10 μg/mL Pam3Cys, 2 ng/mL LPS, 20 μg/mL β-glucan, and β-glucan in combination with Pam3Cys or LPS. Supernatants were collected and analyzed for A) IL-6 and B) TNFα at 24h. Data show results from five independent experiments performed with cells obtained from different donors. *$P < 0.05$ as compared to respective cell culture without the addition of $1,25(OH)_2D_3$.

between TLR2 or TLR4 and Dectin-1. Notably, we found reduced synergistic effects mediated by Dectin-1 when $1,25(OH)_2D_3$ was added to the culture (Figure 4).

Increased *in vivo* $1,25(OH)_2D_3$ led to attenuated inflammatory response to *C. albicans* ex-vivo in human volunteers

To validate our *in vitro* findings, we performed an *ex vivo* study on 15 healthy male volunteers (median age 36, range 28–60 years old). We examined how physiological seasonal variation in sun exposure (through winter, spring, summer, and autumn) could influence host innate immunity against *C. albicans* infections. PBMC from subjects isolated during each season of the year were stimulated with *C. albicans.* It was of great interest to us that we observed a significant drop in IFNγ and IL-17 secretion in spring and summer months, while the IL-10 levels were higher in summer as compared to winter (Figure 5B-5D). These observations correlated with the serum $25(OH)D_3$ concentrations, which doubled in summer compared to winter (Figure 5A). Trends in IL-6 and TNFα production, however, displayed wider variation and were not statistically significant (data not shown).

DISCUSSION

Despite its well-known immunomodulatory effects, nothing to date has been discovered about the effects of vitamin D on the innate immune response to *Candida*. We demonstrate in this study that $1,25(OH)_2D_3$ can significantly modulate host proinflammatory and Th17 response to *C. albicans*, both *in vitro* and *ex vivo*. This biologically active component of vitamin D_3 exerted its effect through modulation of the expression of PRR, recognizing fungi—both TLR2 and TLR4—and C-type lectin receptors, Dectin-1 and MR. Until now, the effect of $1,25(OH)_2D_3$ on CLR has not been recognized.

The constitutive expression of the VDR by human immune cells, including monocytes, macrophages, and lymphocytes, has been well-established (2–4). This is a prerequisite for

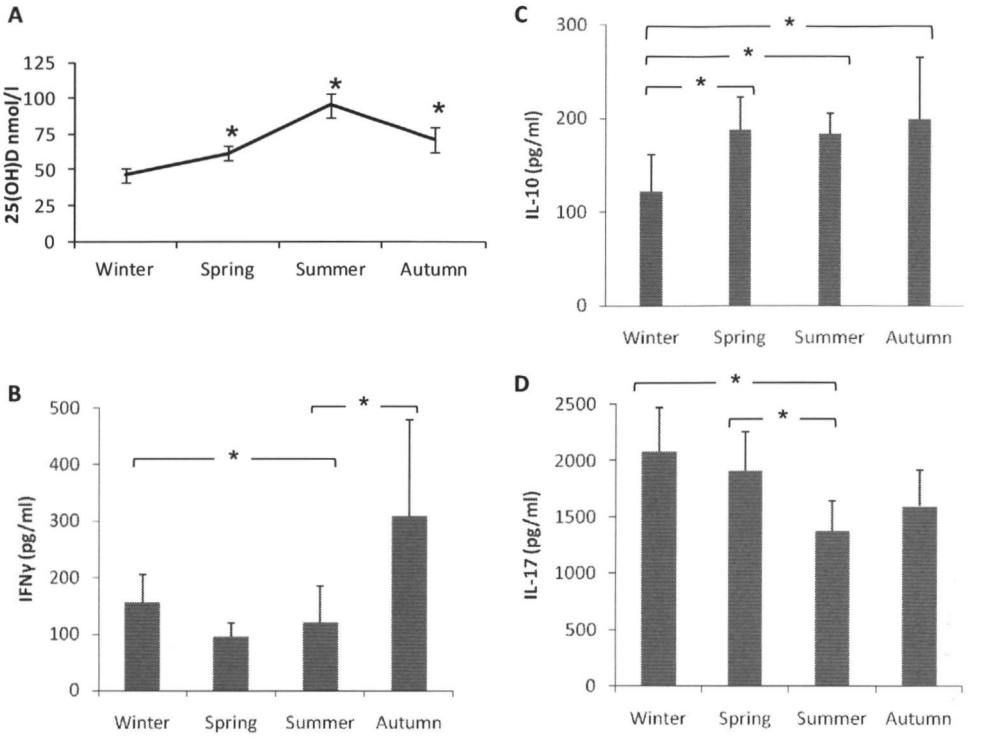

Figure 5. Seasonal variation of host immune response to ex-vivo *Candida albicans* stimulation

A) Mean serum 25(OH)D_3 concentrations of 15 healthy volunteers at different seasons of the year *P < 0.05 as compared to winter. PBMC were stimulated with 10^5 HK *C. albicans* and supernatants collected and analyzed for B) IFNγ, C) IL-10, and D) IL-17 at day 7. Data show results from 15 subjects at the different seasonal time points.

vitamin D_3 to exert any immunomodulatory effect. We have shown that 1,25$(OH)_2D_3$ has a distinct propensity to skew host response to *C. albicans* away from a "proinflammatory" profile through attenuation of TNFα, IL-6, IFNγ as well as production of IL-17. Other researchers have reported inhibition of Th1 cytokine production with 1,25$(OH)_2D_3$, although not in the setting of *Candida* infection and polarization toward a Th2-type response (21–23). In our system using human PBMC stimulated by *C. albicans*, we also observed an increase in IL-10 production in vitamin D presence. We initially hypothesized that this IL-10 might originate from skewing toward Th2 differentiation as seen elsewhere (24;25) or from the potentiation of regulatory T cell function (26;27). However, we were unable to demonstrate any significant shift in the T helper or Treg transcription factors (Tbet, GATA3, Foxp3 or ROR-γt) mRNA expression in our *Candida*-stimulation model under the influence of 1,25$(OH)_2D_3$. Our observation that 1,25$(OH)_2D_3$ had the characteristic capability to suppress IL-6 production, and to some extent, IFNγ also led us to investigate whether the underlying

mechanism was due to novel $1,25(OH)_2D_3$ activity on the respective SOCS3 and SOCS1 regulatory pathways (28). However, we found no evidence of direct or indirect $1,25(OH)_2D_3$-related interference on SOCS regulatory function. Stimulation of SOCS1 and SOCS3 mRNA would have been an anticipated response following the suppression of proinflammatory cytokine production by $1,25(OH)_2D_3$. Slight drops in SOCS1 mRNA expression is a consequence of decreased TLR 2/4 and Dectin-1 signaling rather than the direct effect of $1,25(OH)_2D_3$.

Another mechanism that could account for our observation is vitamin D_3-mediated suppression of PRR expression, followed by modulation of response by host immune cells to *Candida*. $1,25(OH)_2D_3$ indeed diminished surface expression of TLR2 and TLR4, which on follow-up investigation was attributable to its effect on inhibition of upstream TLR2 and TLR4 mRNA transcription. A similar observation had also been reported by Sadeghi et al (29) in the setting of bacterial cell wall components. In addition, $1,25(OH)_2D_3$ had further influence on functioning of other PRRs not previously recognized. Besides TLR2 and TLR4, the C-type lectin receptors Dectin-1 and MR are known to play pivotal roles in triggering the immune signaling cascade upon activation by *C. albicans* (30).We found that $1,25(OH)_2D_3$ activity also exerted inhibition of upstream Dectin-1 and MR protein synthesis. This latter finding was especially significant relative to that of the TLRs, because the extent of $1,25(OH)_2D_3$-induced inhibition of Dectin-1 and MR functioning was suggestively more pronounced than on TLR2 and TLR4. We have also recently reported that *Candida* recognition by CLRs such as Dectin-1 and MR is a more constant feature of antifungal innate immunity than recognition by TLRs (31).

Furthermore, we ascertained the functional consequences of synergistic effects resulted from $1,25(OH)_2D_3$-related suppression of TLR2, TLR4, and Dectin-1 in the setting of *C. albicans* infection. This is important because it has been well-described that "cross-talk" between the TLR2/4 and Dectin-1 is responsible for the synergy of proinflammatory response mounted by the host (32–34). Our use of pure TLR2, TLR4, and Dectin-1 ligands, as well as TLR2/Dectin-1 and TLR4/Dectin-1 ligand combination, illustrated the profound influence that $1,25(OH)_2D_3$ exerted beyond its effect on the individual PRR. The results demonstrated how use of $1,25(OH)_2D_3$ has also led to ablation of the TLR-Dectin-1 synergy, which in the setting of *C. albicans* infection especially, would be very significant. Resultant suppression of MR function most likely further contributes to attenuation of host IL-17 response to *Candida* (35). Hence, the net effect of $1,25(OH)_2D_3$ is a critical shift away from

the proinflammatory response, which would have been anticipated upon *C. albicans* challenge.

We know that production of the vitamin D_3 precursor in the skin depends on UV radiation exposure. A seasonal variation in vitamin D_3 status in temperate climates is widely accepted (36). To validate our *in vitro* findings, we carried out an *ex vivo* study in 15 healthy subjects to investigate the variation in cytokine response to *C. albicans* through the year's four seasons. Compared to winter, during spring and summer months IFNγ and IL-17 response to *Candida* is significantly reduced. This correlates inversely with serum $25(OH)D_3$ levels. Conversely, IL-10 production is higher in summer. Interleukin-6 and TNFα trends were found to be less consistent. This might be attributed to other factors inducing wider biological variation in the *ex vivo* study. Such factors might be UV radiation exposure and the levels of vitamin D_3 necessary to effect a modulation in these cytokine responses. It has been suggested that a higher serum $25(OH)D_3$ level (>100 nmol/L) may be required to sustain immune function (37). Therefore, we ought to be mindful that while certain cytokine trends in the seasonality study correlate with our *in vitro* work, the physiological levels of vitamin D_3 required to comprehensively attenuate the immune responses to *Candida* infection remain to be determined. Nevertheless, our study is the first clear demonstration of seasonal variation in innate immune responses to an important human pathogen.

Research has been ongoing to study the potential role of vitamin D as a therapeutic agent (38). It has been proposed that in invasive fungal infections such as candidiasis and aspergillosis, disease pathology may be attributable to an exaggerated or dysregulated host inflammatory response that results in undue tissue damage (39;40). While a proinflammatory-type immune response will be desirable at disease onset, an unnecessarily prolonged hyperinflammatory phenotype may be averse to outcome. Likewise, Th1 and especially Th17 responses are known to exacerbate and often cause autoimmune phenomena (41;42). Hence, given the immunomodulatory profile of $1,25(OH)_2D_3$, it is tempting to speculate that in specific clinical settings, $1,25(OH)_2D_3$ may have a role in modulating and regulating any unnecessary and prolonged inflammatory response generated by the host. Also, the significant effect of seasonal variation on T helper responses makes it tempting to speculate whether vaccination programs during winter months may be more efficient than those during the summer period. Although speculative at the moment, research on this subject is certainly needed. An encouraging answer would have far-reaching implications for health policy.

In conclusion, we have shown that $1,25(OH)_2D_3$ plays a novel role for the modulation of innate host response to *C. albicans*, by modifying the proinflammatory cytokine responses both *in vitro* and *ex vivo*. By exerting its influence on almost all the major PRRs involved in *Candida* recognition and immune signaling, namely TLR2, TLR4, Dectin-1, and MR, its anti-inflammatory effect is profound in *Candida* infection. Seasonal variation of cytokine responses seems notably important. This opens up a new area of clinical research. We expect that the epidemiological and clinical consequences of our findings could be significant. Future studies are warranted.

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Regulation of Cytokine Responses by Seasonality of Vitamin D Status in Healthy Individuals

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ABSTRACT

The immune modulating capacity of vitamin D_3 is well-recognized. Ultraviolet (UV) exposure determines production of vitamin D_3 *in vivo* and varies through the course of the year, especially in temperate regions. However, it is not known whether the human innate immune response differs due to seasonality. To validate the seasonal effects of vitamin D_3, the effect of $1,25(OH)_2D_3$ on peripheral blood mononuclear cells (PBMC) cytokine response was first determined *in vitro*. $1,25(OH)_2D_3$ decreased interleukin (IL)-6 and tumour necrosis factor (TNF)α release by PBMC stimulated with tripalmitoyl-S-glycerylcysteine (Pam3Cys) or lipopolysaccharide (LPS). Subsequently, *ex vivo* stimulation studies were performed in 15 healthy volunteers through the course of the four seasons of the year. PBMC were isolated and stimulated with Toll-like receptor (TLR)2 and TLR4 ligands Pam3Cys and LPS, respectively. Circulating concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ were higher during summer ($P < 0{\cdot}05$) and a down-regulation of TLR4-mediated IL-1β, IL-6, TNFα, interferon (IFN)γ and IL-10 production in summer was observed compared to winter ($P < 0{\cdot}05$). The variation in cytokine response upon TLR2 (Pam3Cys) stimulation was moderate throughout the four seasons. The repressed cytokine production during the summer months could be explained partly by the reduced cell membrane expression of TLRs. Physiological variation in vitamin D_3 status through the four seasons of the year can lead to alteration in the innate immune responses. Elevated vitamin D_3 level *in vivo* is associated with down-regulation of cytokine response through diminished surface expression of pattern recognition receptors.

INTRODUCTION

The conventional role of vitamin D_3 is that of bone homeostasis and calcium metabolism. Recently, vitamin D_3 has been described to exhibit immunomodulatory effects with the uncovering of vitamin D receptors (VDR) expression on activated $CD4^+$ and $CD8^+$ T lymphocytes and antigen presenting cells (APC) such as monocytes, dendritic cells and macrophages (1-3). The biologically active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), has been shown to influence the differentiation and function of both the innate and adaptive immune cell types and to augment cytokine production differentially (4-6). $1,25(OH)_2D_3$ has been implicated in various immune-mediated diseases, and investigation into its therapeutic potential in autoimmune diseases and infections are ongoing (7,8).

A major source of vitamin D_3 in the body comes from sun exposure (9). Exposure to ultraviolet (UV)B light (290-315 nm) results in the first step of vitamin D_3 biosynthesis, causing 7-dehydrocholestrol to form previtamin D_3 in the skin (10). Previtamin D_3 undergoes a spontaneous thermal isomerization into vitamin D_3. Vitamin D_3 is subsequently hydroxylated into 25-hydroxyvitamin D_3 ($25(OH)D_3$) by 25-hydroxylase in the liver. $25(OH)D_3$ is further converted by 1α-hydroxylase (CYP27B1) in the kidney into the biologically active metabolite, $1,25(OH)_2D_3$. A seasonal variation in vitamin D_3 status in temperate climates is well known (11;12). There has been concern regarding the risk of vitamin D insufficiency among populations residing at higher latitudes where solar radiation during certain periods of the year is inadequate for sufficient cutaneous vitamin D synthesis (13). Epidemiological studies have implicated seasonality and geographical variation in UV radiation as contributing factors to the prevalence of autoimmune disorders such as multiple sclerosis (MS), rheumatoid arthritis and type 1 diabetes (14). It has been reported that the prevalence of MS rises with increasing latitude (15). The season of birth has been implicated in MS occurrence and type 1 diabetes (16-18). Collectively, these data suggest that sun exposure and vitamin D_3 levels are candidate risk-modifying factors in certain autoimmune diseases.

On a similar note, it is widely perceived that there is seasonality for influenza as well as viral upper respiratory tract infections (URTI), and wintertime vitamin D insufficiency is said to be an important contributory factor (19-21). An inverse association between serum $25(OH)D_3$ levels and incidence of URTI has been demonstrated (21;22). A significant reduction in the risk of developing viral URTI was attributed to serum $25(OH)D_3$ levels of more than 30 ng/ml

(75 nmol/l). In other studies, this benefit was conveyed by a higher 25(OH)D_3 level of 40 ng/ml (100 nmol/l) (20;23). Currently, clinical studies evaluating the potential benefits of vitamin D supplementation in reducing the occurrence of seasonal influenza in adults has not been conclusive (24;25), although it seems to be protective in children (26). In a double-blind randomized placebo-controlled trial involving school children (aged 6–15 years), supplementation with 1200 IU cholecalciferol daily during winter reduced influenza A infections significantly.

Despite this wealth of data supporting an immunomodulatory role of vitamin D, no information is available on whether the innate immune response of healthy individuals can vary due to physiological variation in vitamin D_3 store during different seasons of the year. In this study, we investigated whether seasonal variation in vitamin D_3 in the body is associated with differential Toll-like receptor (TLR)2 and TLR4-mediated immune response in healthy subjects living in a temperate region. Such a study would enhance our knowledge on the circumstances determining susceptibility to certain diseases as dictated by the immune-modifying effects of vitamin D_3 *in vivo*.

MATERIALS AND METHODS

Reagents

TLR2 ligand lipopeptide (S)-(2RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys4-OH, tripalmitoyl-S-glycerylcysteine (Pam3Cys) was purchased from EMC Microcollections (Tübingen, Germany). TLR4 ligand lipopolysaccharide (LPS; Esvherichia coli serotype 055:B5) was purchased from Sigma Chemical Co (St Louis, MO, USA). An extra purification step of LPS was performed as described previously (27). 1,25$(OH)_2D_3$ was purchased from Fluka Biochemika, Sigma-Aldrich (Missouri, MI, USA) and dissolved in absolute ethanol. The reagents were all prepared just before commencement of the seasonal study and stored as aliquots at −70°C for single use only. This was conducted to ensure uniformity of the respective stimuli used for the study.

Stimulation assays

The study was approved by the Ethical Committee on Human Experimentation of the Radboud University Nijmegen. Written consent was obtained from all participants to the study. Venous blood was drawn into ethylenediamine tetraacetic acid (EDTA) tubes from healthy subjects for *in vitro* experiments. For the *ex vivo* study, 15 healthy male volunteers

(median age 36 years, range 28-60; mean body mass index (BMI) 22·8 kg/m^2, range 20·5-26·2) were recruited and followed up for 1 year. Venous blood was drawn from the subjects during four consecutive seasons: winter (December-February), spring (March-May), summer (June-August) and autumn (September-November) of 2009. On the rare occasions that any of the participants reported being unwell, the experiment was postponed until 1 week post-recovery.

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). Cells were washed twice in saline, counted in a Coulter counter and the number adjusted to 5 × 10^6 cells/ml. A 100 µl volume of PBMC, suspended in culture medium (RPMI-1640 DM; ICN Biomedicals, Costa Mesa, CA, USA) supplemented with 10 µg/ml gentamicin, 10 mM l-glutamine, 10 mM pyruvate and 10% human pooled serum was added to flat-bottomed 96-well plates (Greiner, Alphen a/d Rijn, the Netherlands).

For the *in vitro* study to validate the effects of vitamin D_3, PBMC were preincubated with 100 nm 1,25$(OH)_2D_3$ for 30 min, followed by addition of TLR2 ligand (Pam3Cys 10 µg/ml), TLR4 ligand (LPS 10 ng/ml) or RPMI-1640 (as unprimed control). As with the *ex vivo* study, PBMC were stimulated with similar concentrations of Pam3Cys and LPS. In addition, other concentrations tested *ex vivo* include Pam3Cys 1 µg/ml and LPS 1 and 100 ng/ml. Cell cultures were incubated in a 37°C, 95% humidity, 5% CO_2 incubator. The culture supernatants were collected after 24 h (or 48 h for interferon (IFN)-γ) of incubation as appropriate and stored at −20°C until cytokine assay.

Flow cytometry

Cells were analysed phenotypically by five-colour flow cytometry (Coulter Cytomics FC 500; Beckman Coulter, Fullerton, CA, USA) using Coulter Epics Expo 32 software. PBMC as well as whole blood (after cell lysis) was used for flow cytometry analysis after. Cells were washed with PBS with 0·2% bovine serum albumin (BSA) before being labelled with fluorochrome-conjugated antibodies (mAb). After incubation for 20 min at room temperature in the dark, cells were washed twice to remove unbound antibodies and analysed. For cell surface staining, the following mAbs were used: TLR2-fluorescein isothiocyanate (FITC) (TL2·1) and TLR4-phycoerythrin (PE) (HTA125), both from eBioscience (San Diego, CA, USA).

Cytokine measurements

Interleukin (IL)-6, IL-1β, IL-10 and IFNγ concentrations were measured using commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits (Pelikine Compact; CLB, Amsterdam, the Netherlands) according to the manufacturer's instructions. Human tumour necrosis factor (TNF)α was measured by a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA). Detection limits were 8 pg/ml (IL-10 and IFNγ), 16 pg/ml (IL-6), 20 pg/ml (IL-1β) and 40 pg/ml (TNFα).

Vitamin D_3 measurement

Serum 25(OH)D_3 was determined using high-performance liquid chromatography (HPLC) with ultraviolet detection, after prior extraction on small SepPak columns. Tritiated 25(OH)D_3, collected from the HPLC system during the passage of the UV peak, was used to correct for procedural losses. Serum 1,25$(OH)_2D_3$ was measured using a radioreceptor assay (RRA) with prior extraction and chromatographic prepurification with correction for recovery as described previously (28).

Statistical analysis

Results were pooled and analysed using spss 16·0 statistical software. Data given as means ± standard error of the mean (S.E.M) and Wilcoxon's signed-rank test was used to compare differences between groups (unless otherwise stated). The level of significance was set at $P < 0{\cdot}05$.

RESULTS

1,25$(OH)_2D_3$ resulted in suppression of proinflammatory cytokine response *in vitro*

First, we carried out LPS and Pam3Cys stimulation on PBMC with the addition of 100 nM 1,25$(OH)_2D_3$. After 24 h, IL-6 secretion was reduced by 53% and 29% upon Pam3Cys and LPS stimulation, respectively, in the cells treated with 1,25$(OH)_2D_3$ (Figure 1A). A significant drop in TNFα production by 17% (Pam3Cys stimulation) and 35% (LPS stimulation) was also observed with 1,25$(OH)_2D_3$ at 24 h (Figure 1B). Having demonstrated that 1,25$(OH)_2D_3$ had the capacity to modulated IL-6 and TNFα response *in vitro*, and with the understanding that physiological vitamin D_3 levels in the body may vary through the seasons, we validate these observations *in vivo* in a cohort of healthy volunteers.

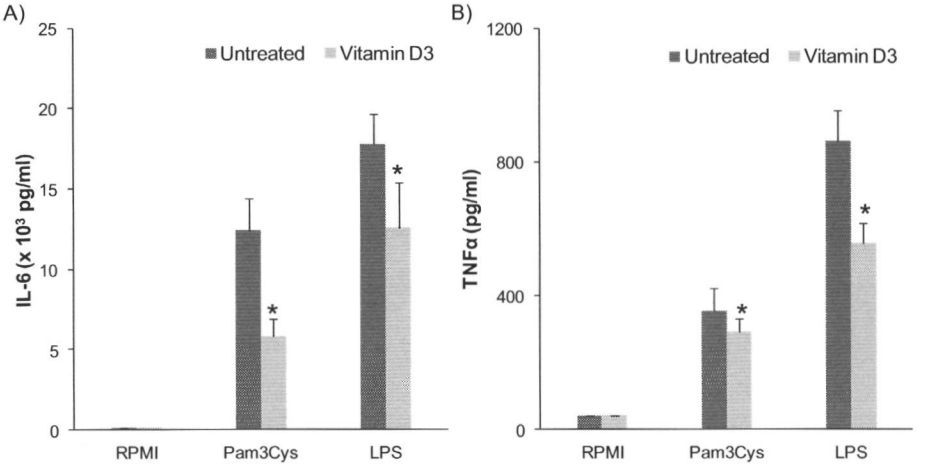

Figure 1. The effect of vitamin D_3 on cytokine responses after *in vitro* stimulation
Peripheral blood mononuclear cells (PBMC) were preincubated in the presence or absence (carrier) of 100 nM $1,25(OH)_2D_3$ and thereafter stimulated with 10 µg/ml tripalmitoyl-S-glycerylcysteine (Pam3Cys) or 10 ng/ml lipopolysaccharide (LPS). After 24 h, A) interleukin (IL)-6 and B) tumour necrosis factor (TNF)α were measured in the supernatant. Data show results from five independent experiments performed with cells obtained from different donors. *P < 0·05 compared to respective untreated cell culture without the addition of $1,25(OH)_2D_3$.

Serum $25(OH)D_3$ and $1,25(OH)_2D_3$ was increased significantly in summer

We determined the serum levels of $25(OH)D_3$ and $1,25(OH)_2D_3$ levels in 15 healthy male volunteers through winter (December-February), spring (March-May), summer (June-August) and autumn (September-November). The amount of sunlight in the study region varied with the seasons (Figure 2A). The total duration of sunlight in a month prior to vitamin D_3 levels and cytokine measurement was 103 h and 240 h in winter and summer, respectively. The median $25(OH)D_3$ level increased steadily from 43 nmol/l in winter to spring and doubled to 89 nmol/l in summer, with a drop again in autumn (Figure 2B). The median serum $1,25(OH)_2D_3$ levels raised significantly from 219 pmol/l in winter to 237 pmol/l in summer (Figure 2C).

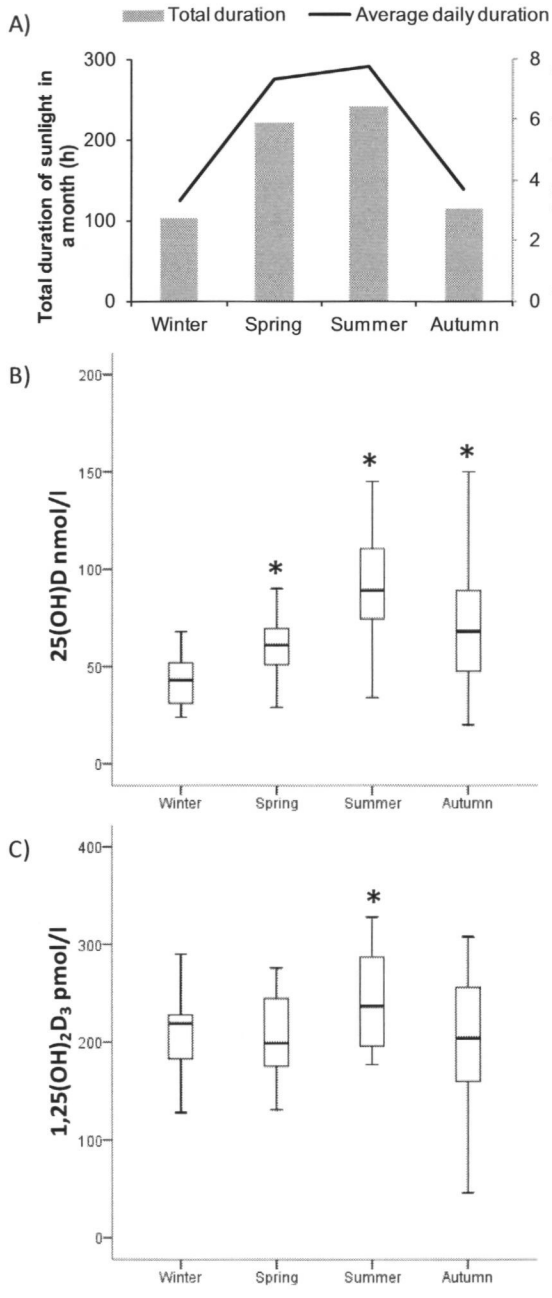

Figure 2. Variation in serum vitamin D_3 levels throughout the four seasons of the year

A) Total duration of sunlight in the study region 4 weeks prior to serum vitamin D_3 concentration assay (source: the Royal Netherlands Meteorological Institute). Median serum B) $25(OH)D_3$ and C) $1,25(OH)_2D_3$ concentrations of 15 healthy volunteers at different seasons of the year. *P < 0·05 compared to winter.

TLR4 (LPS)-mediated cytokine response with variation in vitamin D_3 levels

Increased serum vitamin D_3 level in summer was associated with a significant drop in TNFα (64%), IL-6 (33%), IL-1β (59%) and IFNγ (46%) production when compared against winter

(Figure 3A-D). IL-10 production was also reduced by 55% ($P < 0{\cdot}05$) during summer compared to winter (Figure 3E). These observations show that elevated serum vitamin D_3 levels are associated with attenuation of the host inflammatory response after engagement of TLR4.

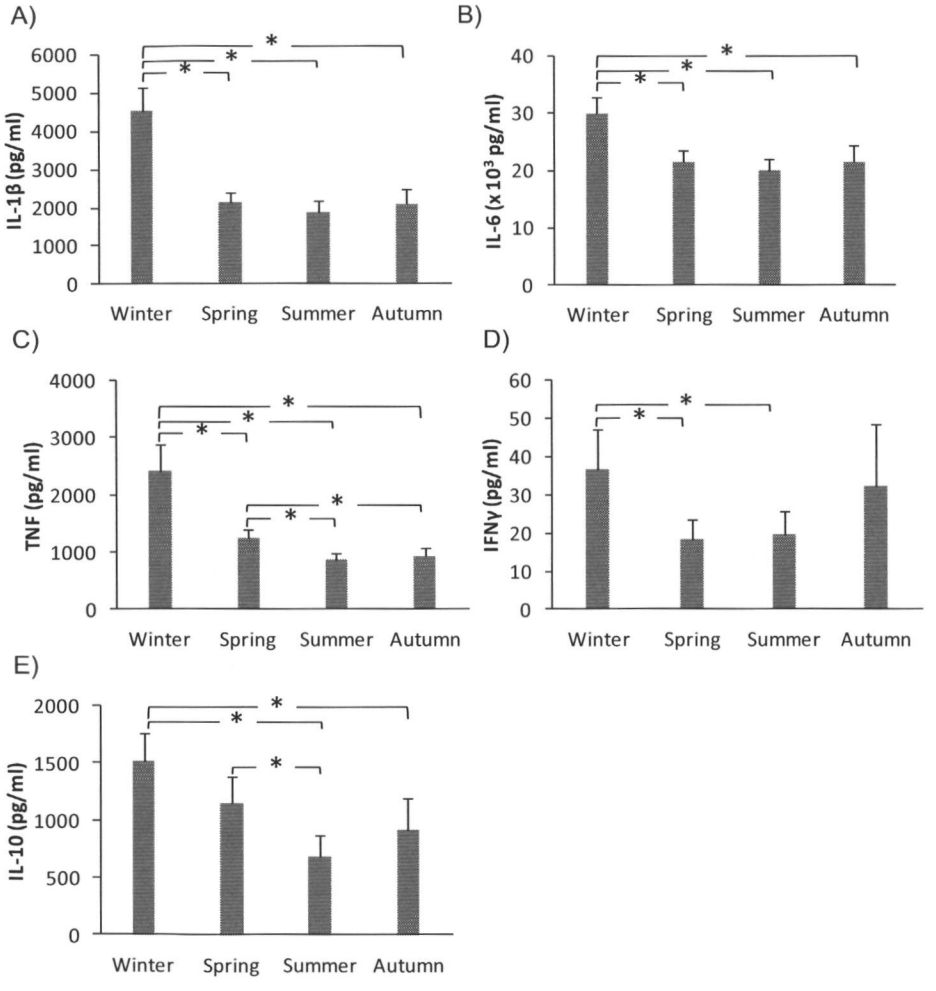

Figure 3. Variation in cytokine responses to lipopolysaccharide (LPS) stimulation during the four seasons of the year

Peripheral blood mononuclear cells (PBMC) were isolated from 15 healthy volunteers during each of the four seasons and stimulated with 10 ng/ml LPS. A) Interleukin (IL)-1β, B) IL-6, C) tumour necrosis factor (TNF)α, D) interferon (IFN)γ and E) IL-10 were measured. Data show results from 15 healthy donors. *$P < 0{\cdot}05$ compared among the respective seasons.

TLR2 (Pam3Cys)-mediated cytokine response with variation in vitamin D_3 levels

We also determined the regulation of cytokine production upon Pam3Cys stimulation of PBMC. After 24 h of stimulation, a modest drop in TNFα and IL-1β production by 27% ($P < 0{\cdot}05$) and 22% ($P > 0{\cdot}05$), respectively, was seen during summer compared to winter (Figure 4A and C). The variation in IL-6 and IL-10 (Figure 4B and D) production was limited throughout the course of the four seasons. The seasonal variation of TLR2-mediated host cytokine response was more limited compared to TLR4-mediated responses.

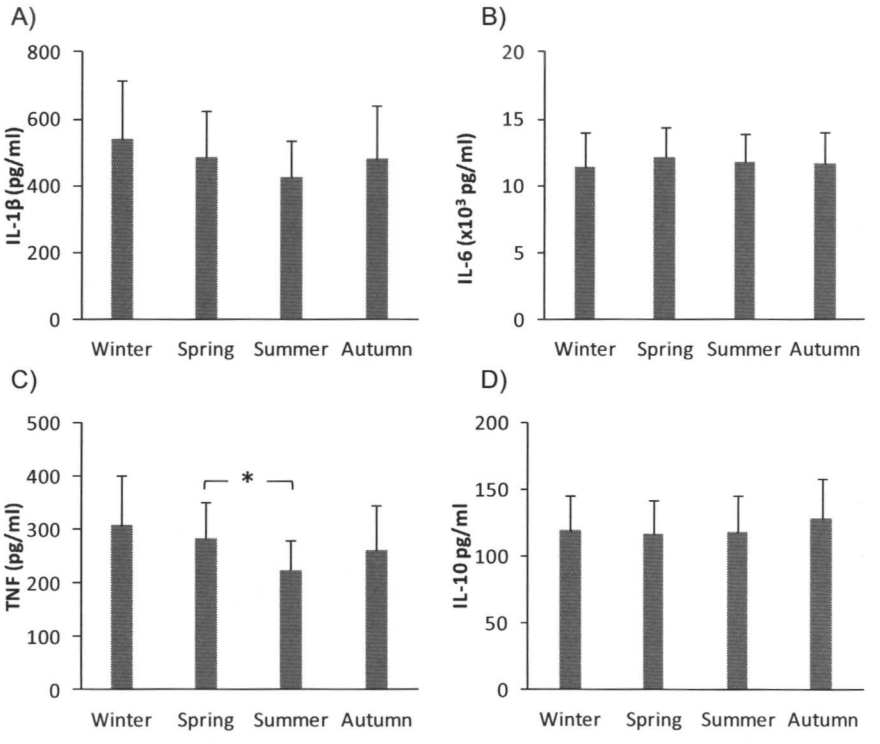

Figure 4. Variation in cytokine responses to tripalmitoyl-S-glycerylcysteine (Pam3Cys) stimulation during the four seasons of the year

Peripheral blood mononuclear cells (PBMC) were isolated from 15 healthy volunteers during each season and stimulated with 10 μg/ml Pam3Cys. A) Interleukin (IL)-1β, B) IL-6 and C) tumour necrosis factor (TNF)α and D) IL-10 were measured. Data show results from 15 healthy donors. *$P < 0{\cdot}05$ compared among the respective seasons.

Reduced TLR2 and TLR4 expression could explain the suppression of cytokine responses in summer

We have found recently that $1,25(OH)_2D_3$ had the capacity to modulate TLR2 and TLR4 expression *in vitro* (6). Therefore, we investigated whether the attenuated proinflammatory

cytokine responses observed in our *ex vivo* experiments was indeed the result of an altered pattern recognition receptors (PRR) expression. Using flow cytometric analysis, we found that the expression of both TLR2 and TLR4 on monocytes was reduced during summer when compared to winter (Figure 5A and B).

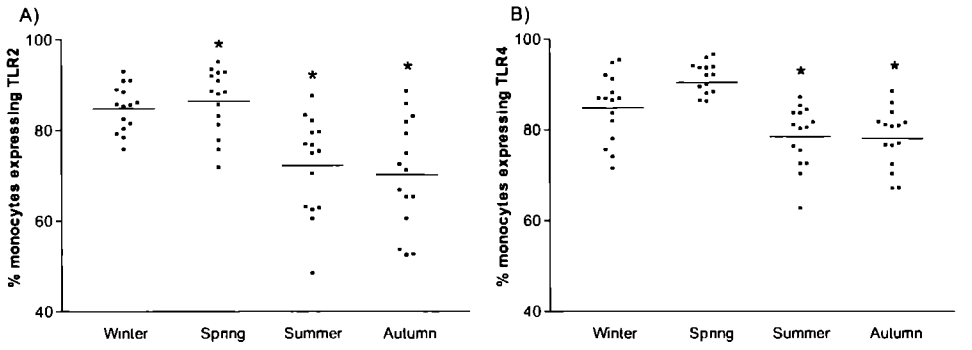

Figure 5. Expression of A) Toll-like receptor (TLR)2 and B) TLR4 on the cell membrane of monocytes
Peripheral blood mononuclear cells (PBMC) were isolated from 15 healthy volunteers during each season and analysed for the respective markers using flow cytometry as gated on monocytes. Data show results from 15 healthy donors. *P < 0·05 compared to winter.

DISCUSSION

Much interest has been shown recently in the immunomodulatory capacity of vitamin D_3 and the role it plays in health and diseases. While biosynthesis upon UVB exposure serves as the main source of vitamin D_3 in the body (9), it is not known if seasonal variation in vitamin D_3 store arising from a fluctuating solar exposure can impact the innate immune response. We show for the first time that, relative to winter levels, there was a physiological elevation in vitamin D_3 store during summer and this led to down-regulation in proinflammatory cytokine production, particularly when stimulated via the TLR4-mediated signalling pathway.

$25(OH)D_3$ is the major circulating form of vitamin D_3 and its concentration is used commonly as an indicator of vitamin D_3 status (29), whereas $1,25(OH)_2D_3$ is the biologically active form of vitamin D_3. BMI has been shown to be related inversely to vitamin D_3 levels (30). This confounder has been excluded due to the fact that none of the volunteers in the study was obese (BMI > 30 kg/m^2). Here, we found that the difference between winter (December-February) and summer (June-August) $25(OH)D_3$ levels to be 46 nmol/l. As expected, serum $1,25(OH)_2D_3$ concentrations were also higher in summer compared to winter. The body

vitamin D_3 levels correlated with the amount of sunlight in the study region. In our cohort of 15 subjects residing at 52°N from the Equator, we were hence able to demonstrate an evident variation of vitamin D_3 status throughout the four seasons. Other studies have also demonstrated such a difference in young adults residing along similar latitudes at 40–50°N (11;12;31). Epidemiological studies have suggested that wintertime vitamin D insufficiency may account for the seasonal variation in incidence of viral respiratory tract infections (19;20;22). As such, it is most appropriate to look further into how the host immune responses vary during the different seasons.

Looking specifically at TLR2 and TLR4-mediated response, as main pattern recognition receptors are involved, we first demonstrated that *in vitro* $1,25(OH)_2D_3$-treated cells produced less IL-6 and TNFα. In line with the modulatory effects of vitamin D_3, a general down-regulation of cytokine production was found during the summer months when the serum vitamin D_3 levels were elevated, especially when PBMC from the volunteers were challenged with LPS. A fundamental aspect to be taken into account is that the physiological up-regulation of vitamin D_3 levels by solar radiation in our study differs from the vitamin D_3 doses employed in the *in vivo* and *in vitro* studies. This could explain why the effect on IL-6 production was less evident with Pam3Cys in our *ex vivo* experiment, although there was a 50% reduction in the *in vitro* set-up. Conversely, we saw a similar reduction in TNFα production both *in vitro* and *ex vivo* following ligation of TLR2.

One of the key targets of $1,25(OH)_2D_3$ is the $CD4^+$ T lymphocytes. $1,25(OH)_2D_3$ is known to suppress the secretion of IFNγ and IL-2, while enhancing IL-4, IL-5 and IL-10 production (32;33). By inhibiting the secretion of IFNγ, $1,25(OH)_2D_3$ limits antigen presentation and recruitment of other T cells, thereby down-regulating the proinflammatory response. Conversely, $1,25(OH)_2D_3$ promotes the production of the anti-inflammatory cytokines IL-4 and IL-10, thus shifting the balance towards a T helper type 2 (Th2) or regulatory T cells (Treg) phenotype (34;35). Of note, a major difference from some of these previous *in vitro* and mice studies is that our *ex vivo* data show both the proinflammatory and anti-inflammatory cytokines being down-regulated in summer. Again, the concentrations of $1,25(OH)_2D_3$ used *in vitro* and the actual physiological levels in the body may account for this apparent discordance. In contrast, there have also been recent reports of $1,25(OH)_2D_3$ suppressing both IFNγ and IL-10 in $CD4^+$ T lymphocytes (36). In a similar experimental set-up using LPS stimulation, Matilainen et al pointed out that the impaired IL-10 production induced by $1,25(OH)_2D_3$ was time-dependent and not sustainable. They reported that while

IL-10 secretion from monocytes was suppressed by $1,25(OH)_2D_3$ during the initial 8 h, there was a rebound up-regulation at 48 h (37).

We attempted to study the mechanisms responsible for the down-regulation of the proinflammatory cytokine response during summer. An explanation could be that $1,25(OH)_2D_3$ induces down-regulation of TLR expression, and this may account for the attenuated response after stimulation with TLR2 and TLR4 ligands (6;38). Indeed, we found a reduced surface expression of TLR2 and TLR4 associated with elevated vitamin D_3 stores during summer which persisted throughout autumn. Through modification of TLR2 and TLR4 expression, $1,25(OH)_2D_3$ had the propensity to limit cytokine response following ligation of the receptors. Similarly, Sadeghi et al has demonstrated *in vitro* that $1,25(OH)_2D_3$ down-regulates TLR2 and TLR4 mRNA and protein expressions on monocytes, and this resulted in an impaired TNFα production upon LPS challenge (38). Furthermore, they concluded that this effect was VDR-dependent by showing that the down-regulation of TLR expression was reversed by a vitamin D receptor antagonist. However, we note from our *ex vivo* experiments that the reduction of TLR2 expression during summer was accompanied by a corresponding drop in TNFα production following stimulation with Pam3Cys, but this trend was less evident for IL-6.

A still unresolved question arising from ongoing bench and clinical research has been the serum vitamin D_3 concentrations needed to elicit an optimal immune response (39). The present results also suggest that there may be differences in the intracellular pathways leading to TNFα and IL-6 production (40), and these pathways may be modulated differentially by vitamin D_3 concentration *in vivo*. Nevertheless, overall it seems remarkable here that our observations streaming from the physiological changes in vitamin D_3 levels show similar trends correlating TLR expression and cytokine production compared to those from an *in vitro* setting.

In the present study, we assessed a homogeneous study population (healthy, adult males with normal BMI) to establish if and how host cytokine response vary with seasons. Unique to previous *in vitro* and *in vivo* studies examining the role of vitamin D_3, our data showed how innate immune responses can be influenced by the physiological variation of serum vitamin D_3 levels during the four seasons of the year. It would be prudent to validate these observations in a larger and more diverse population cohort to identify any possible

differences in cytokine responses among the extreme of ages and different genders. Nevertheless, with these results in mind, it is tempting to hypothesize whether response to vaccination programmes could be different when carried out during winter or summer. A recent study conducted in a small cohort of prostate cancer patients suggested that there was a trend towards a better serological response rate against among patients with higher serum $25(OH)D_3$ levels (41). However, the $25(OH)D_3$ level delineating such a difference was not reported. Conversely, a clinical trial in healthy volunteers showed that co-administration of $1,25(OH)_2D_3$ with influenza vaccine did not affect serological response against H1N1, H3N2 or influenza B antigens (42). Further research is needed to validate this hypothesis, as this would have far-reaching implications for implementation of preventive health policies. These data may also provide better insight into previous epidemiological findings regarding the prevalence of autoimmune diseases and infections, which have been attributed to seasonal variation in vitamin D_3 status. In a step further, the season of birth has been implicated with the occurrence of MS and insulin-dependent diabetes mellitus (16–18). In addition to that, higher serum $25(OH)D_3$ levels have been associated with lower relapse risk in MS, occurring in a dose-dependent linear fashion (43). Hypothetically, a heightened proinflammatory response in utero during wintertime may have important connotation on eventual development of MS and insulin-dependent diabetes mellitus. Further studies are warranted to validate this by looking at cytokine responses during pregnancy across the four seasons and assessing the therapeutic benefits of vitamin D supplementation during pregnancy.

In conclusion, we have demonstrated for the first time that variations in innate immune response exist throughout the four seasons of the year. In summer, elevated serum vitamin D_3 levels are associated with an attenuated cytokine-producing capacity attributable to a suppressed expression of TLR2 and TLR4. This harbours potentially important implications for our understanding of disease epidemiology and implementation of vitamin D_3 supplementation in temperate regions during winter.

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Seasonal Variation in Vitamin D_3 Levels is Paralleled by Changes in the Peripheral Blood Human T Cell Compartment

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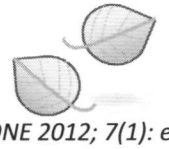

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ABSTRACT

It is well-recognized that vitamin D_3 has immune-modulatory properties and that the variation in ultraviolet (UV) exposure affects vitamin D_3 status. Here, we investigated if and to what extent seasonality of vitamin D_3 levels are associated with changes in T cell numbers and phenotypes. Every three months during the course of the entire year, human PBMC and whole blood from 15 healthy subjects were sampled and analyzed using flow cytometry. We observed that elevated serum $25(OH)D_3$ and $1,25(OH)_2D_3$ levels in summer were associated with a higher number of peripheral $CD4^+$ and $CD8^+$ T cells. In addition, an increase in naïve $CD4^+CD45RA^+$ T cells with a reciprocal drop in memory $CD4^+CD45RO^+$ T cells was observed. The increase in $CD4^+CD45RA^+$ T cell count was a result of heightened proliferative capacity rather than recent thymic emigration of T cells. The percentage of regulatory T cell (Treg) dropped in summer, but not the absolute Treg numbers. Notably, in the Treg population, the level of forkhead box protein 3 (Foxp3) expression was increased in summer. Skin, gut and lymphoid tissue homing potential was increased during summer as well, exemplified by increased CCR4, CCR6, CLA, CCR9 and CCR7 levels. Also, in summer, $CD4^+$ and $CD8^+$ T cells revealed a reduced capacity to produce proinflammatory cytokines. In conclusion, seasonal variation in vitamin D_3 status *in vivo* throughout the year is associated with changes in the human peripheral T cell compartment and may as such explain some of the seasonal changes in immune status which has been observed previously. Given that the current observations are limited to healthy adult males, larger population-based studies would be useful to validate these findings.

INTRODUCTION

Vitamin D_3 is traditionally associated with bone homeostasis and calcium metabolism. The extra-renal synthesis of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) by macrophages and other immune cells has re-invented the role of vitamin D_3. In recent years, research efforts were also focused on understanding the immune-modulatory properties of vitamin D_3. 1,25-dihydroxyvitamin D_3 has been shown to influence the growth and differentiation of both the innate and adaptive immune cells, as well as their functions such as cytokine production (1-3). As such, there has been much interest to identify its therapeutic potential in autoimmune or inflammatory diseases.

Sources of vitamin D_3 include dietary uptake (primarily fatty fish and cod liver oil) as well as cutaneous biosynthesis from ultraviolet (UV)B exposure causing 7-dehydrocholestrol to form previtamin D_3 in the skin. Vitamin D_3 is subsequently hydroxylated into 25-hydroxyvitamin D_3 ($25(OH)D_3$) by 25-hydroxylase in the liver. 25-hydroxyvitamin D_3 is further hydroxylated by 1α-hydroxylase in the kidney into the biologically active metabolite, $1,25(OH)_2D_3$ (4). The main source of vitamin D_3 derives from UVB-induced vitamin D_3 production, accounting for 80 to 90% of circulating vitamin D_3 (5). The seasonal fluctuation in vitamin D_3 status in temperate and cold climates with reduced sunlight exposure during certain periods of the year is thought to be responsible for the high prevalence of vitamin D_3 insufficiency among populations residing at higher latitudes (6). Low wintertime vitamin D_3 levels have been found partly accountable for the seasonal peak in influenza and URTI occurrence (7-9). Moreover, reduced sun exposure and vitamin D_3 status have been identified as risk factors for the development of autoimmune diseases. Epidemiological studies have implicated seasonality of birth as well as geographical variation in UV radiation and serum vitamin D_3 levels as contributing factors to the prevalence of multiple sclerosis and insulin-dependent diabetes mellitus (10-15).

T cells are known targets for $1,25(OH)_2D_3$ since they express vitamin D receptor (16;17). Upon T cell activation, the expression of vitamin D receptor (VDR) is up-regulated, suggesting an important functional role for vitamin D_3 in adaptive immunity. Both human *in vitro* and animal models revealed that vitamin D_3 can suppress proinflammatory T helper (Th)1 and Th17 cytokine responses (18;19), while enhancing the production of interleukin (IL)-4, IL-5 and IL-10, thereby promoting a Th2 and regulatory T cell (Treg) phenotype (20;21). Indeed, accumulating evidence supports the notion that vitamin D_3 could favourably

influence the course of certain autoimmune pathology by increasing the number of Treg (13;15). In addition, chemokine receptors expression is a determining factor in migration and localization of T lymphocytes during physiological and inflammatory responses (22;23); $1,25(OH)_2D_3$ has been demonstrated to affect the homing capacity of the peripheral $CD4^+$ T cell population *in vitro* and in an animal model (24;25).

Taken together, the involvement of $1,25(OH)_2D_3$ in the dynamics of T cell compartment warrants further investigation. Previously, we have found a down-regulation of Toll-like receptor (TLR)4-mediated proinflammatory cytokines production in association with an elevated vitamin D_3 status in summer (26). However, our current knowledge on the immunomodulatory role of vitamin D_3 conveys limited information on how the adaptive immune response of healthy individuals varies in response to physiological changes in vitamin D_3 status *in vivo* during the different seasons of the year. Intrigued by the strong epidemiological association between vitamin D_3 deficiency and autoimmunity, and the proposed effects of $1,25(OH)_2D_3$ on Treg, we investigated whether there is a seasonal variation in the composition of the peripheral T cell pool and the circulating Treg. A potential modification in these parameters may provide a better understanding on how sun exposure and vitamin D_3 can act as candidate risk-modifying factors in certain autoimmune disorders.

MATERIALS AND METHODS

Study subjects

Fifteen healthy male volunteers (median 36 years old, range 28-60; mean BMI 22.8 kg/m^2, range 20.5 – 26.2) were recruited and followed up for one year. Body mass index (BMI) has been shown to be inversely related to vitamin D_3 levels (27). We have eliminated this confounder from our study since none of the 15 volunteers was obese (BMI > 30 kg/m^2). Venous blood was drawn from the subjects every three months, at the end of four consecutive seasons in 2009; February in winter, May in spring, August in summer and November in autumn. On the rare occasions that a participant reported on being unwell, the experiment would be postponed until one week post-recovery.

Ethics statement

The study was approved by the Ethical Committee on Human Experimentation of the Radboud University Nijmegen. A written consent was obtained from all participants in the study.

Flow cytometry

Cells were phenotypically analyzed by five-color flow cytometry (Coulter Cytomics FC 500, Beckman Coulter, Fullerton, USA) using Coulter Epics Expo 32 software. PBMC as well as whole blood (after red cell lysis) were used for flow cytometric analysis. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). Cells were washed with PBS with 0.2% bovine serum albumin (BSA) before being labeled with fluorochrome-conjugated antibodies (mAb). After incubation for 20 minutes at room temperature, in the dark, cells were washed twice to remove unbound antibodies and analyzed. For cell surface staining, the following mAb were used: CD127 PC5- or PC7-labeled (RDR5; eBioscience, Uithoorn, The Netherlands), CD25-PE (M-A251), CD25-APC (2A3) CD45RA-FITC (HI100), CCR4-PC7 (1G1), CCR6-PE (11A9), CLA-FITC (HECA-452) (all from BD Biosciences, Breda, The Netherlands), CD3-ECD (UCHT1), CD4 ECD- or PC7-labeled (SFCI12T4D11), CD4-PC5 (13B8.2), CD8-ECD (SFCI21Thy2D3), CD8-PC5 (B9.11), CD27-PC5 (1A4CD27), CD45RA-ECD (2H4LDH11LDB9) CD45RO-ECD (UCHL1) (all from Beckman Coulter, Mijdrecht The Netherlands), CCR7-FITC (150503), CCR9-PE (112509) (both from R&D Systems, Minneapolis, USA), CD27-FITC (M-T271), CD45-PE (T29/33), CD45RA-PE (4KB5) (both from Dako, Glostrup, Denmark) and CD31 Alexa Fluor® 488 (WM59) (BioLegend, San Diego, USA). Appropriate isotype control mAbs were used for gate settings. The live gate was set based on the forward angle light scatter (FSCs) and the side angle light scatter (SSCs), and Annexin-V/PI staining.

For intracellular staining of FoxP3 and Ki-67, cells were fixed and permeabilized using Fix and Perm reagent (eBioscience) according to the manufacturer's recommendations. The following mAb were used for staining: anti-FoxP3 FITC- or PE- labeled (FCH101; eBioscience), anti-Ki-67-FITC (B56, BD Biosciences).

Intracellular staining of cytokines was performed after 4 hours stimulation with PMA (12.5 ng/ml) and ionomycin (500 ng/ml) in the presence of Brefeldin A (5 µg/ml; Sigma, Zwijndrecht, The Netherlands). Cells were fixed and permeabilized using Fix and Perm reagent (eBioscience) according to the manufacturer's recommendations. The following mAb were used for staining: anti-IFNγ-PC7 (4S.B3), anti-IL-17-Alexa Fluor® 647 (eBIO64DEC17) (both from eBioscience), and anti-IL-2-PE (MQ1-17H12) (BD Bioscience).

Vitamin D_3 measurement

Serum 25(OH)D_3 was determined using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, after prior extraction on small SepPak columns as previously

described (28). Tritiated $25(OH)D_3$, collected from the HPLC system during passage of the UV peak, was used to correct for procedural losses. Serum $1,25(OH)_2D_3$ was measured using a radioreceptor assay (RRA) with prior extraction and chromatographic pre-purification with correction for recovery as previously described (29). For $25(OH)D_3$, the within run precision was 2.6% at 69 nmol/L and between run precision was 6.2% at 69 nmol/L. For $1,25(OH)_2D_3$, the within run precision was 10.6% at 115 pmol/L and between run precision was 17.2% at 69 nmol/L.

Statistical analysis

Results were pooled and analyzed using SPSS 16.0 statistical software. Data given as means + SEM and the Analysis of Variance (ANOVA) was performed to assess overall variation. Where the ANOVA indicated a significant difference ($P < 0.05$), the Friedman test using Graphpad PRISM software (Graphpad Prism Inc., version 4, CA, USA) was used to compare differences between groups (unless otherwise stated). The level of significance was set at $P < 0.05$.

RESULTS

Serum $25(OH)D_3$ and $1,25(OH)_2D_3$ levels are increased during summer

First, we determined serum concentrations of both $25(OH)D_3$ and $1,25(OH)_2D_3$ in 15 healthy volunteers (median 36 years old, range 28-60) through winter (December to February), spring (March to May), summer (June to August) and autumn (September to November). The median concentration of $25(OH)D_3$ varied between the four seasons and was doubled from 43 nmol/L in winter to 89 nmol/L in summer (Figure 1A). Also, the median serum concentration of $1,25(OH)_2D_3$ raised significantly from 219 pmol/L in winter to 237 pmol/L in summer (Figure 1B). These observed trends paralleled the amount of sunlight in the study region. Likewise, there is considerable seasonal difference in ultraviolet B (UVB) radiation in the study region (30). The total duration of daylight in a month prior to vitamin D_3 measurement were 103 hours and 240 hours in winter and summer respectively, which worked out to an average daily duration of 3.3 hours in winter and 7.7 hours in summer (Figure 1C).

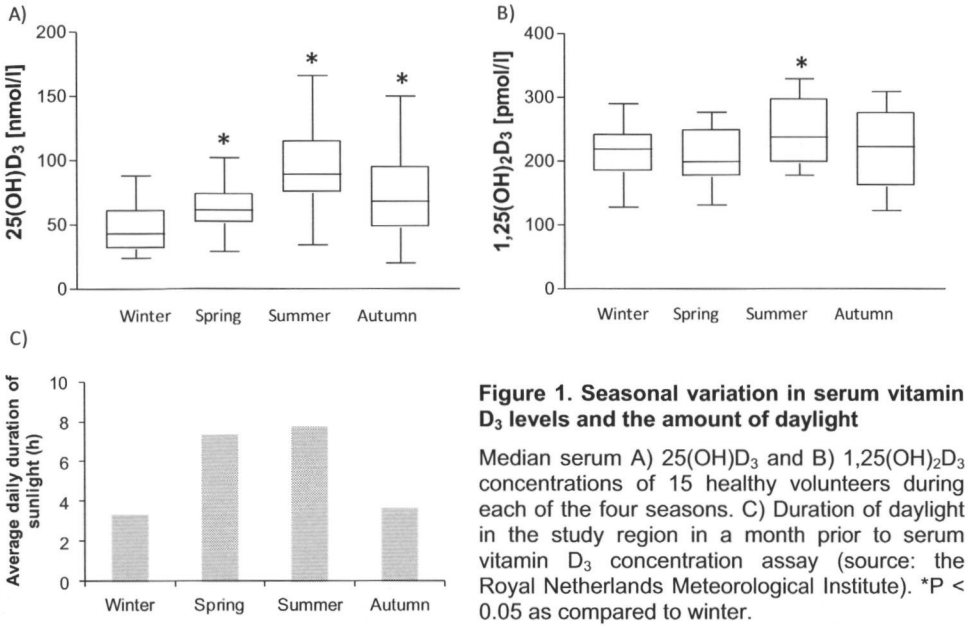

Figure 1. Seasonal variation in serum vitamin D_3 levels and the amount of daylight

Median serum A) $25(OH)D_3$ and B) $1,25(OH)_2D_3$ concentrations of 15 healthy volunteers during each of the four seasons. C) Duration of daylight in the study region in a month prior to serum vitamin D_3 concentration assay (source: the Royal Netherlands Meteorological Institute). *P < 0.05 as compared to winter.

Seasonal variation in peripheral blood T cell subset numbers associated with vitamin D_3 levels

Next, we investigated whether seasonal variation in vitamin D_3 status was associated with changes in the peripheral T cell pool, by performing flowcytometric analysis on blood samples obtained during the different seasons of the year (Figure S1). In spring and summer months when serum vitamin D_3 levels were elevated, the percentage as well as the absolute $CD4^+$ T cell counts were significantly raised as compared to winter (Figure 2A). For $CD8^+$ T cells, this effect was less outspoken (Figure 2B).

The composition and size of the naïve and memory T cell pools are regulated by cytokines and T cell receptor (TCR) signalling from contact with major histocompatibility complex (MHC). Naïve T cells predominately express CD45RA and memory T cell express CD45RO. Interestingly, during spring and summer, we observed a relative increase in the percentage of $CD4^+CD45RA^+$ T cells (Figure 2C), with a corresponding drop in $CD4^+CD45RO^+$ T cell percentage (Figure 2D). Also, absolute $CD4^+CD45RA^+$ T cell counts were increased in spring and summer months, while the number of $CD4^+CD45RO^+$ T cells was not significantly changed. To investigate whether the increase in peripheral $CD4^+CD45RA^+$ T cells as observed in spring and summer could be attributed to recent thymic emigration or a higher proliferative capacity; we stained cells with Ki-67 and CD31. Ki-67 is a nuclear protein

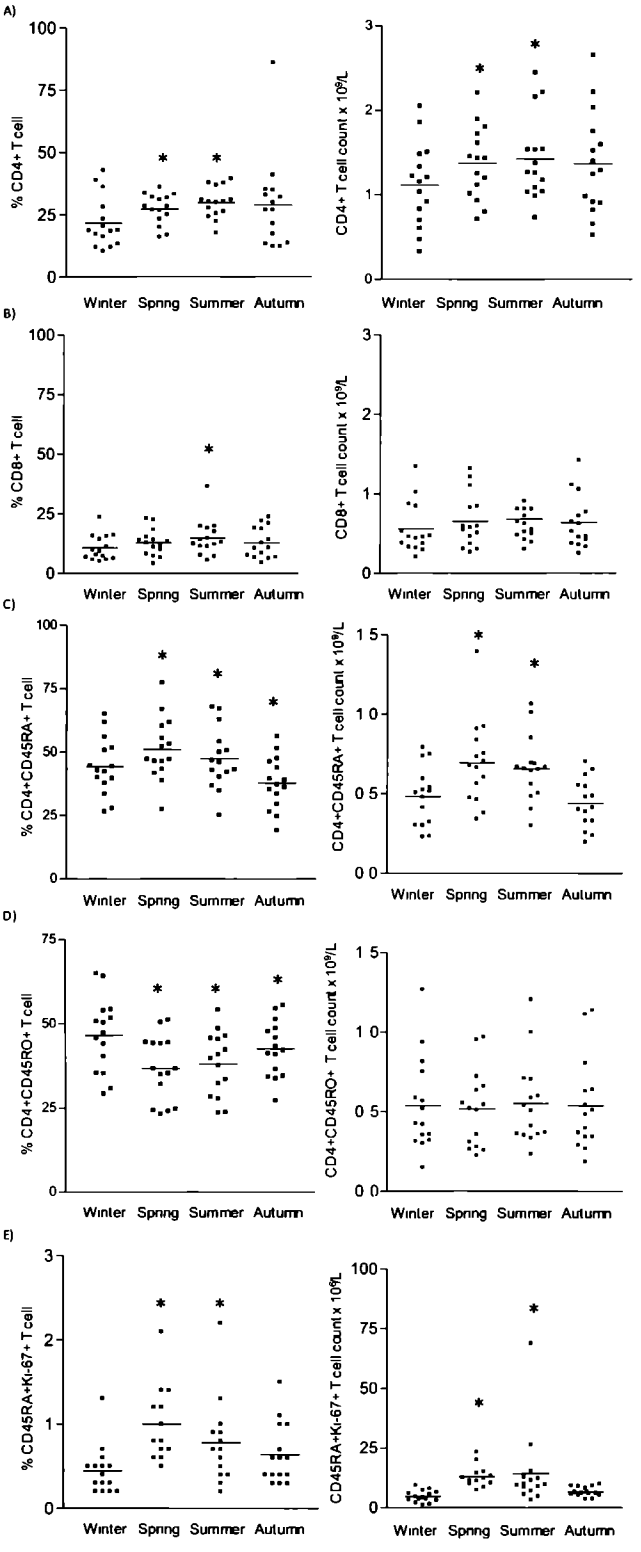

Figure 2. Peripheral T cell (subset) numbers throughout the four seasons

A) Percentage (of live gate) and absolute numbers of $CD4^+$ T cells. B) Percentage (of live gate) and absolute numbers of $CD8^+$ T cells, over time C) Percentage (within $CD4^+$ T cells) and absolute counts, of $CD4^+CD45RA^+$ T cells. D) Percentage (within $CD4^+$ T cells) and absolute counts, of $CD4^+CD45RO^+$ T cells. E) Percentage and absolute counts of Ki-67-expressing $CD4^+CD45RA^+$ T cells. Whole blood samples obtained from 15 healthy volunteers during each season were analyzed for the respective markers using flow cytometry. Ki-67 analysis was performed on PBMC. Data show results of viable cells from 15 healthy donors. *P < 0.05 as compared to winter.

associated with cellular proliferation, while CD31 has been used as a marker for recent thymic emigrants (31). In spring and summer, an increased Ki-67-expressing population was found within the $CD4^+CD45RA^+$ T cells (Figure 2E). On the other hand, there were no significant differences in both the frequency of $CD4^+CD45RA^+$ T cell expressing CD31 as well as their level of expression between winter and summer (data not shown).

The increase in vitamin D_3 levels found in summer, as compared to winter was paralleled by a reduction in the percentage of $CD25^{hi}CD127^-$ Treg within the $CD4^+$ T cell population (Figure 3A), however the absolute Treg numbers were not associated with the variation in vitamin D_3 levels. Of note, the level of expression (mean fluorescence intensity, MFI) of Foxp3 by the peripheral Treg population was increased in summer (Figure 3B).

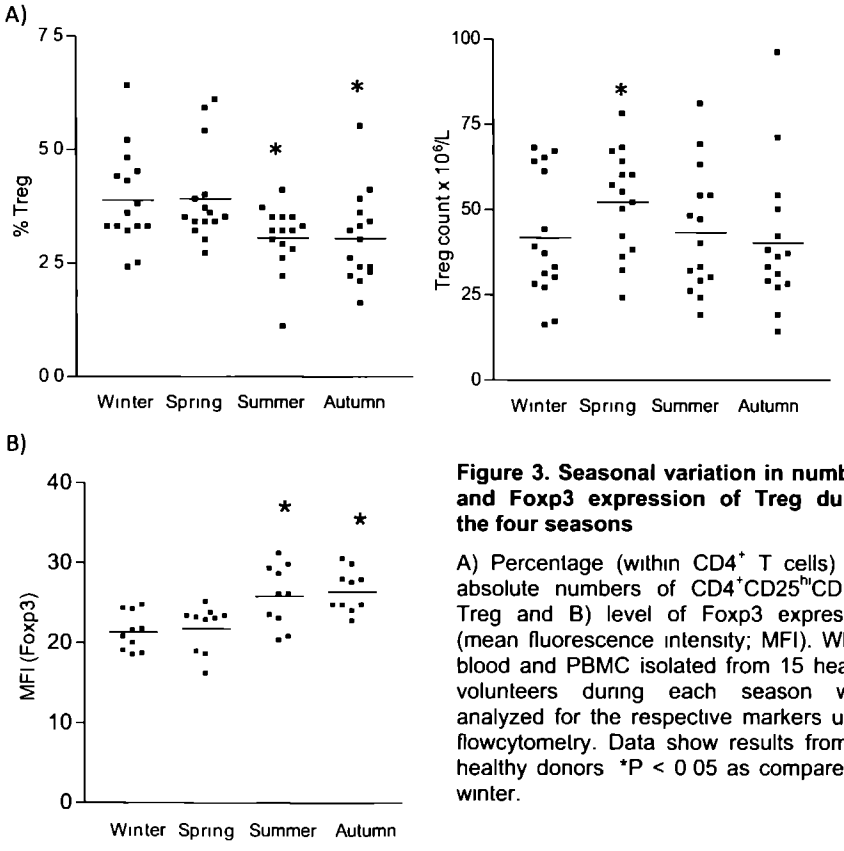

Figure 3. Seasonal variation in numbers and Foxp3 expression of Treg during the four seasons

A) Percentage (within $CD4^+$ T cells) and absolute numbers of $CD4^+CD25^{hi}CD127^-$ Treg and B) level of Foxp3 expression (mean fluorescence intensity; MFI). Whole blood and PBMC isolated from 15 healthy volunteers during each season were analyzed for the respective markers using flowcytometry. Data show results from 15 healthy donors *P < 0 05 as compared to winter.

Seasonal variation in homing potential of peripheral blood $CD4^+$ T cells

Peripheral T cell trafficking is regulated by specific chemokine receptors which are selectively expressed by the various T cells subsets. As $1,25(OH)_2D_3$ has been

demonstrated to affect the homing capacity of the peripheral $CD4^+$ T cell population *in vitro* and *in vivo,* we wondered if we could detect seasonal variation in homing receptors expression. We looked at the expression of homing markers on $CD4^+$ T cells, as well as more specifically on the Treg population, and included chemokine receptors associated with migration to the skin (CCR4, CCR6 and CLA), gut (CCR9) and lymphoid tissues (CCR7).

In summer, an increased skin homing potential of $CD4^+$ T cells was observed compared to winter, given that the percentage of $CD4^+$ T cells expressing CCR4 and CCR6 (Figure 4A and 4B) was significantly increased together with elevated expression levels (MFI) of CCR4, CCR6 and CLA (Figure 4A-C). Also, the percentage of $CD4^+$ T cells expressing the gut homing marker CCR9 was increased in summer, as well as the level of expression (Figure 4D). Similar observations were seen in the expression level of the chemokine receptor associated with lymphoid tissue homing, CCR7 (Figure 4E).

The skin homing potential of the regulatory T cell subset mirrored that of the whole peripheral $CD4^+$ T cell population. In summer Treg displayed a heightened skin homing potential as seen by a significantly increased frequency of CCR4-expressing Treg (Figure 5A), and higher expression levels (MFI) of CCR4, CCR6 and CLA (Figure 5A-C), when compared to winter. The level of expression (MFI) of chemokine receptors involved in gut homing, CCR9 (Figure 5D) and lymphoid tissue homing, CCR7 (Figure 5E) were also increased in summer.

Reduced proinflammatory cytokine production by peripheral blood T cells in summer

Intrigued by the increased $CD4^+$ T cell numbers in spring and summer, we also looked at functional characteristics of the cells by examining the cytokine-producing capacity of $CD4^+$ T cells using intracellular cytokine staining for interferon (IFN)γ, IL-2 and IL-17. There was no significant effect on the percentage of IFNγ-producing $CD4^+$ T cells (Figure 6A), but the level of production on a per cell basis (MFI) was lowered in summer ($p<0.05$). The percentages of IL-2 and IL-17- secreting $CD4^+$ T cells were reduced in summer (Figure 6B and 6C), with unchanged levels of production on a per cell basis (MFI).

For $CD8^+$ T cells we also found lowered levels of IFNγ production (MFI) from spring to autumn (Figure 6D). The percentage of $CD8^+$ T cells producing IL-2 was significantly reduced from spring to autumn (Figure 6E); expression levels were increased during spring.

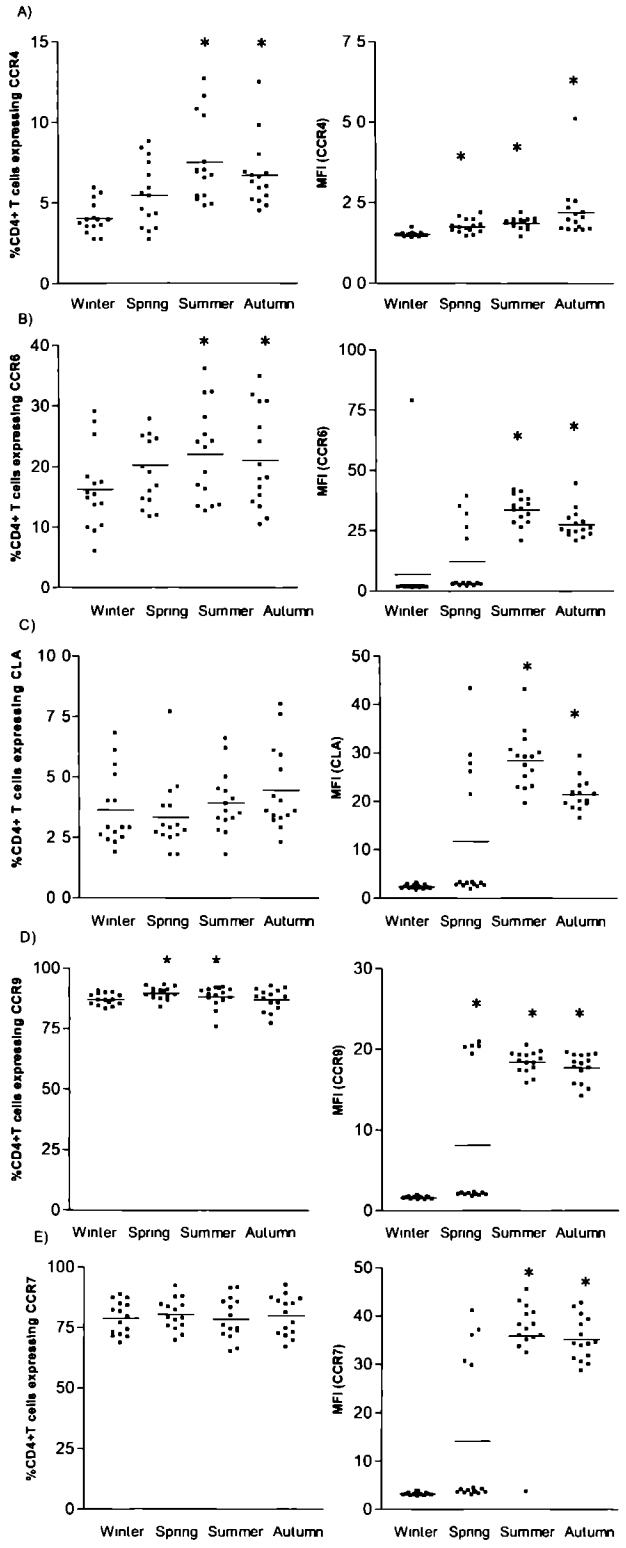

Figure 4. Skin, lymphoid tissue and gut homing receptor expression on CD4$^+$ T cells

Percentage and level of expression (MFI) of A) CCR4, B) CCR6, C) CLA, D) CCR7 and E) CCR9 by CD4$^+$ T cells during the different seasons of the year. Whole blood from 15 healthy volunteers during each season was analyzed for the respective markers using flow cytometry. Data show results from 15 healthy donors. *P < 0.05 as compared to winter.

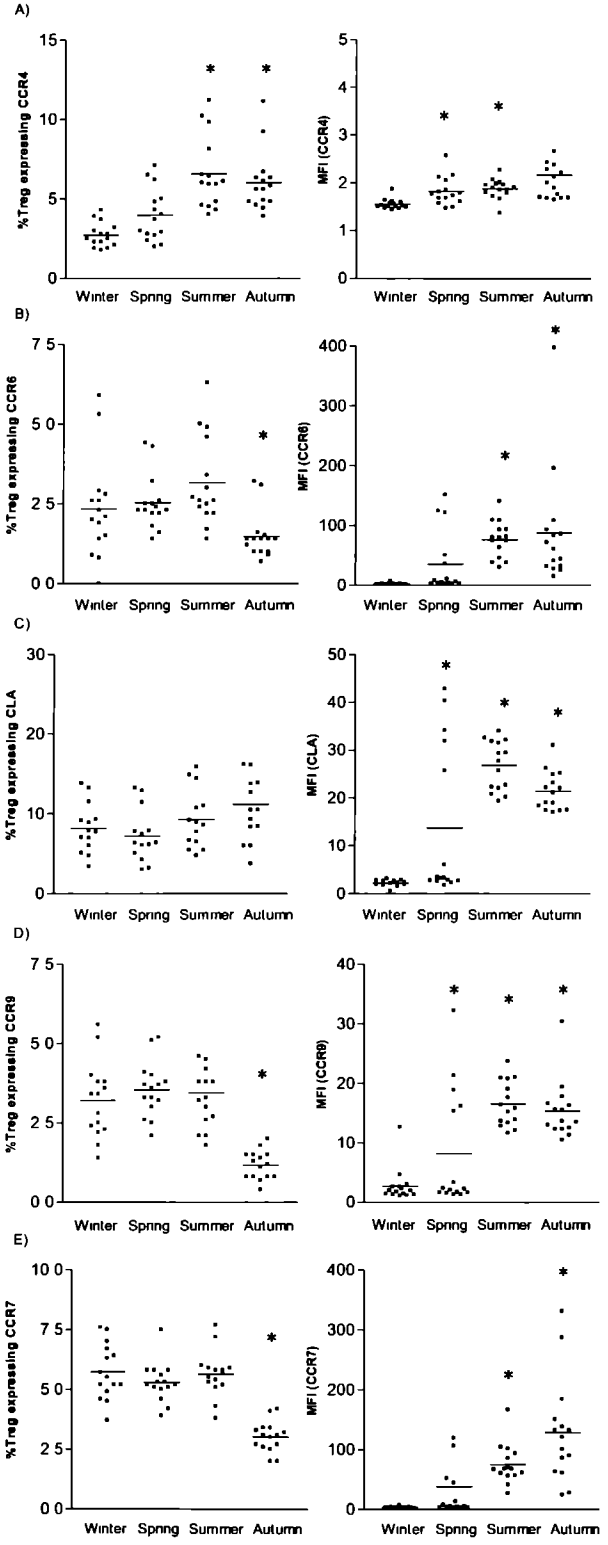

Figure 5. Skin, lymphoid tissue and gut homing receptor expression on $CD4^{+}CD25^{hi}CD127^{-}$ regulatory T cells

Percentage of Treg (within $CD4^{+}$ T cells) and their level of expression (MFI) of A) CCR4, B) CCR6, C) CLA, D) CCR7 and E) CCR9 during the four seasons of the year. Whole blood from 15 healthy volunteers during each season was analyzed for the respective markers using flow cytometry. Data show results from 15 healthy donors *P < 0.05 as compared to winter.

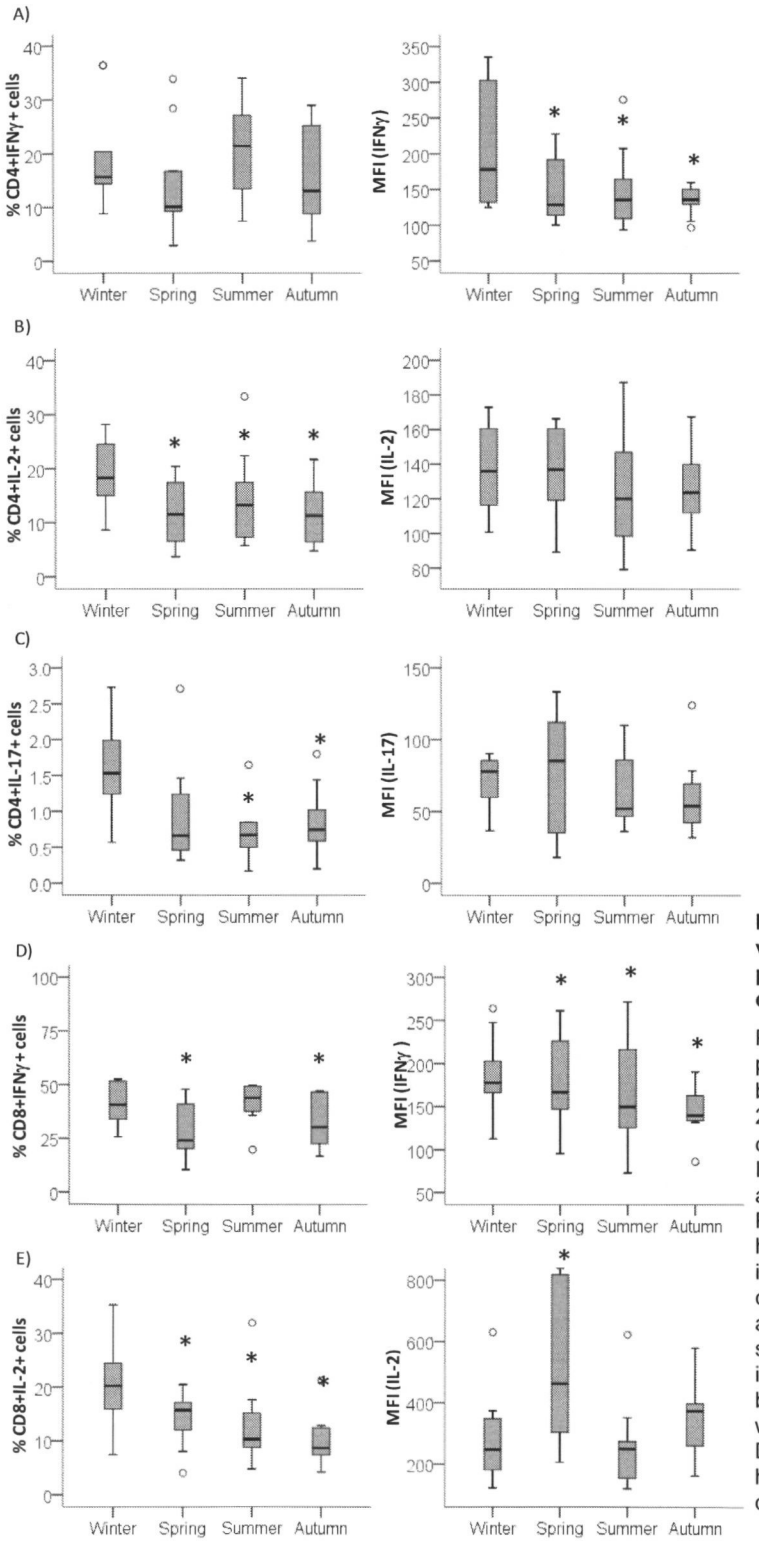

Figure 6. Seasonal variation in cytokine profile of $CD4^+$ and $CD8^+$ T cells

Percentage and the level of production on a per cell basis (MFI) of A) IFNγ, B) IL-2 and C) IL-17 by $CD4^+$ T cells; and of D) IFNγ and E) IL-2 by $CD8^+$ T cells analyzed by flow cytometry. PBMC isolated from 10 healthy volunteers and intracellular staining for cytokines was performed after the cells were stimulated with PMA plus ionomycin in the presence of brefeldin A. $CD4^+$ T cells were defined as $CD3^+CD8^-$. Data show results from 10 healthy donors. *P < 0.05 as compared to winter.

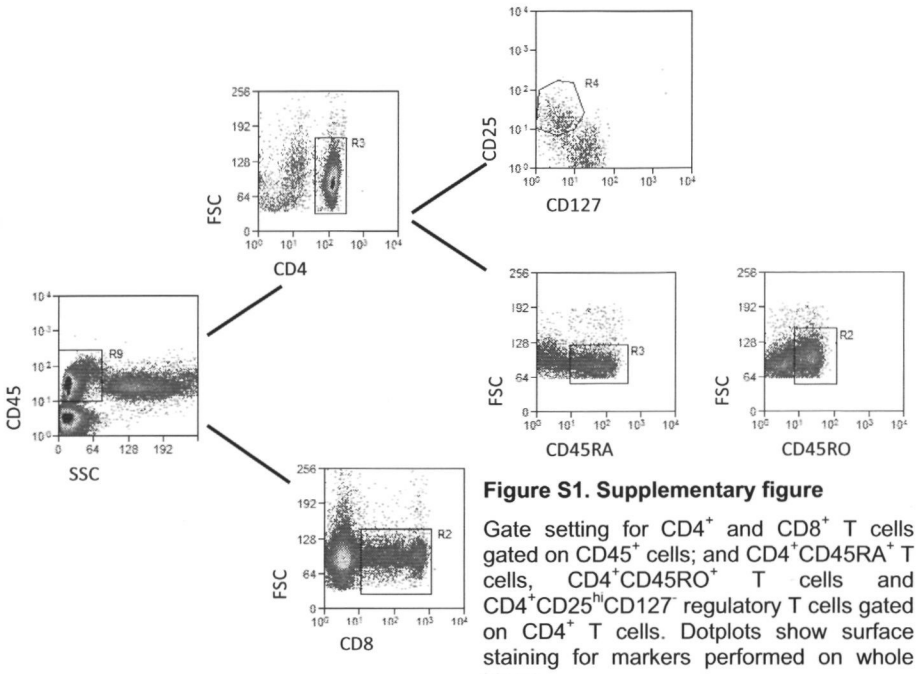

Figure S1. Supplementary figure

Gate setting for $CD4^+$ and $CD8^+$ T cells gated on $CD45^+$ cells; and $CD4^+CD45RA^+$ T cells, $CD4^+CD45RO^+$ T cells and $CD4^+CD25^{hi}CD127^-$ regulatory T cells gated on $CD4^+$ T cells. Dotplots show surface staining for markers performed on whole blood.

DISCUSSION

There is growing evidence that vitamin D_3 plays a pivotal role in infections and autoimmune diseases. Whilst UV-induced vitamin D_3 production serves as the main source of vitamin D_3 in the body (5), it is not apparent whether seasonal variation in vitamin D_3 can impact T cell immunity. We show for the first time that physiological elevation in vitamin D_3 concentrations during summer is paralleled by changes in the peripheral T cell composition, with a notable shift in the naïve and memory $CD4^+$ T cell balance as a consequence of increased proliferation of naïve $CD4^+CD45RA^+$ T cells.

By virtue of its stability and long half-life, $25(OH)D_3$ is the vitamin D metabolite that best reflects the vitamin D_3 status (32). Here, we found a significant difference between winter (December to February) and summer (June to August) $25(OH)D_3$ levels. Serum $1,25(OH)_2D_3$ concentrations were also higher in summer as compared to winter. In our cohort of 15 subjects residing at 52°N from the Equator, this variation correlated with the amount of sunlight and ultraviolet B radiation received in the study region. Vitamin D_3 insufficiency at high latitudes has been implicated in the prevalence of autoimmune diseases such as multiple sclerosis and insulin-dependent diabetes (33;34). Therefore, we investigated

whether the peripheral T cell compartment might vary with physiological changes in vitamin D_3 status throughout the year.

We found higher percentages of peripheral $CD4^+$ and $CD8^+$ T cells concomitant with a heightened vitamin D_3 status during summer. Interestingly, we observed a higher proportion of $CD4^+CD45RA^+$ naïve T cells in the spring/summer months with a corresponding drop in the percentage, but not in the absolute number, of $CD4^+CD45RO^+$ memory T cells. When investigated further, the expansion of $CD4^+CD45RA^+$ naïve T cells resulted from an increased proliferative capacity as seen by a higher absolute cell count and an increased population expressing the proliferative marker, Ki-67. One of the key targets of $1,25(OH)_2D_3$ are the $CD4^+$ T cells. *In vitro*, $1,25(OH)_2D_3$ inhibits T cell proliferation (35;36). Though few studies examined the differential effects on naïve and memory T cells, the inhibitory effect has been found to be more pronounced in the memory T cell compartment (37).

$1,25(OH)_2D_3$ exerts a marked inhibitory effect on cells of the adaptive immune system and it has been consistently described that $1,25(OH)_2D_3$ inhibits cytokines such as IFNγ (21;38) and IL-17, as well as IL-2 (19;39), both under *in vitro* conditions and in animal models. Our data reveal that in healthy adult males residing at 52°N from the Equator, the percentages of IL-17- and IL-2-producing $CD4^+$ T cells were down-regulated in summer and the IFNγ production on a per cell basis (MFI) in both $CD4^+$ and $CD8^+$ T cells was also reduced.

Regulatory T cells are characterized by a constitutively high expression of the transcription factor, Foxp3. We observed that, although the percentage of peripheral Treg was lower in summer as compared to winter, there was no correlation between absolute numbers of Treg and vitamin D_3 levels. This is in concert with findings of Smolders et al, who failed to detect a correlation between Treg numbers and serum $25(OH)D_3$ levels in patients with multiple sclerosis (40). Interestingly, they did find that higher $25(OH)D_3$ levels were associated with improved suppressive function. This fits our data on increased expression of Foxp3 in the Treg during summer. Morales-Tirado et al reported that *in vitro*, $1,25(OH)_2D_3$ enhanced Treg function by increasing the expression of Foxp3 and that this was shown to be associated with modulation of cell cycle progression by vitamin D_3 (41).

T cell migration is determined by the presence of specific selectins, chemokine receptors and integrins. Homing receptors are selectively expressed and regulated in different T cell subsets (23;42). Our results are suggestive of a vitamin D_3 associated up-regulation of skin-, gut- and lymphoid tissue- homing expression on $CD4^+$ T cells, including Treg. Although not previously described in the context of physiological variation, $1,25(OH)_2D_3$ has been reported to influence certain skin homing markers in human T cells. *In vitro*, it has been shown that addition of $1,25(OH)_2D_3$ resulted in induction of CCR10, inhibition of CLA, but not CCR4 and CCR6 expression (38;43). In our study, we found that during summer an increased frequency of CCR4-expressing cells as well as an increased level of expression (MFI) of CCR4, CCR6 and CLA. These data suggest that in summer $CD4^+$ T cells, including Treg, are better equipped to migrate to the skin. Also, we observed higher levels of CCR9 and thus heightened potential to migrate to the gut. Previously, $1,25(OH)_2D_3$ was described not to affect gut-homing markers (25). However, it should be appreciated that the physiological up-regulation of vitamin D_3 levels by UV light through the skin is likely to yield distinct effects from those obtained through supraphysiological doses employed in these *in vitro* studies.

In the present study, we assessed a homogenous study population (healthy, adult males of normal BMI) to establish if and how the human peripheral T cell compartment varies with the season. Unique to previous *in vitro* and *in vivo* studies examining the role of $1,25(OH)_2D_3$ on T cells, our current results suggest that physiological variation in serum vitamin D_3 levels throughout the four seasons might influence $CD4^+$ and $CD8^+$ T cell homeostasis and homing behavior. Given that serum $25(OH)D_3$ levels can be affected by various factors, our observations warrant future validation in a larger and more diverse population cohort to identify any possible differences in adaptive immune responses among the extreme of ages and different genders. Nevertheless, our data provide insight on previous epidemiological findings regarding the prevalence of certain autoimmune diseases and infections, which have been attributed to seasonal variation in sun exposure and serum $25(OH)D_3$ levels (10-12),(44). Although not as extensively reported as vitamin D_3 status, certain hormones and corticosteroids such as catecholamine and aldosterone seem to vary with seasons as well (45;46). It would be of interest to find out if these factors are associated with changes in immunological characteristics of T cell.

In conclusion, we have demonstrated for the first time the existence of variations in adaptive immunity throughout the four seasons of the year in association with physiological changes in serum $25(OH)D_3$ levels *in vivo*. These novel findings further our understanding on the

seasonal variability between vitamin D_3 and human peripheral T cell composition, and support the basis for conducting larger population-based studies to investigate the benefits of vitamin D_3 supplementation in temperate regions during winter.

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1,25-Dihydroxyvitamin D_3 Inhibits Proliferation but not the Suppressive Function of Regulatory T Cells in the Absence of Antigen-Presenting Cells

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ABSTRACT

Vitamin D_3 is known to induce regulatory T cell (Treg) by rendering antigen presenting cells (APC) tolerogenic, its direct effect on human naturally-occurring Treg is unclear. Here, we investigated if and how 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) can directly affect the proliferation and function of human naturally-occurring Treg *in vitro*. First, we demonstrated that these Tregs express vitamin D receptor that was up-regulated following anti-CD3/CD28-bead stimulation. $1,25(OH)_2D_3$ inhibited proliferation of Treg even when exogenous interleukin (IL)-2 was provided. Tregs were more susceptible to the inhibitory effect of $1,25(OH)_2D_3$ than conventional T cells (Tconv) $1,25(OH)_2D_3$ neither affected the anergic state nor the suppressive function of Treg but induced a subtle increase in IL-10-secreting cells. The cell division inhibiting effect of $1,25(OH)_2D_3$ on Treg was also demonstrated *in vivo* by supplementing vitamin D deficient HIV-1 infected patients with 2000 IU cholecalciferol (vitamin D_3). Increased serum $1,25(OH)_2D_3$ levels were associated with a drop in the number and percentage of Treg which may be attributed to a decrease in proliferating $Foxp3^+$ Treg population. In conclusion, $1,25(OH)_2D_3$ directly affects Treg growth and promotes IL-10 production without apparent effects on activation status and suppressive phenotype whereas *in vivo*, high serum $1,25(OH)_2D_3$ levels are associated with reduced Treg proliferation and a reduced number of Treg.

INTRODUCTION

Vitamin D_3 is mostly associated with its primary role in bone and calcium metabolism, but recent interest hinges on its potential as an immunomodulatory agent. Vitamin D_3 is obtained by dietary intake or by cutaneous previtamin D_3 biosynthesis upon ultraviolet (UV)B exposure. Previtamin D_3 is subsequently hydroxylated into 25-hydroxyvitamin D_3 ($25(OH)D_3$) in the liver and further hydroxylated by 1α-hydroxylase in the kidney into the active metabolite, $1,25(OH)_2D_3$ (1). $1,25(OH)_2D_3$ is the ligand for vitamin D receptor (VDR). Several mononuclear cells including T cells, express VDR (2;3) and can also produce $1,25(OH)_2D_3$ by expressing 1α-hydroxylase (4;5). A role for vitamin D_3 in immune regulation has been demonstrated both *in vivo* and *in vitro;* $1,25(OH)_2D_3$ influences the growth, differentiation and cytokine production of various immune cells (6-8). As a result, $1,25(OH)_2D_3$ has been proposed as a potential immunomodulatory agent in autoimmune diseases such as insulin-dependent diabetes mellitus, multiple sclerosis, inflammatory bowel disease and rheumatoid arthritis (9-11), as well as in cancer (12) and infectious diseases such as HIV infection (13).

Regulatory T cells are characterized by a $CD4^+CD25^{hi}$ phenotype and signature transcription factor, forkhead box protein 3 (Foxp3). They are an integral part of the immune system and are crucial in the regulation of immune homeostasis (14). It has been demonstrated that the regulatory function of $CD4^+CD25^+$ Treg is hampered in autoimmunity, allergy and infectious diseases, indicating that these cells play an important role in immune-mediated pathology. Over the past decade, there has been a streaming amount of evidence on the potential therapeutic application of Treg either in enhancing their regulatory activity in inflammatory diseases such as autoimmunity, allograft rejection, graft versus host disease and allergic diseases, or in blocking their suppressive activity in tumour immunity or vaccine development (15-18).

Treg can either be derived from the thymus, the so-called naturally-occurring Treg (nTreg) or they can be induced *de novo* in the periphery from $CD4^+$ T cells. Vitamin D_3 interferes with the maturation and differentiation of dendritic cells and may induce a so-called tolerogenic phenotype (19;20). This implies that $1,25(OH)_2D_3$ can indirectly potentiate the differentiation of IL-10-producing $CD4^+CD25^+$ regulatory T cells by altering the function of antigen presenting cells (21;22). $1,25(OH)_2D_3$ can also act directly on $CD4^+CD25^-$ T cells to generate $Foxp3^+$ T cells expressing high levels of CTLA-4 that are capable of immune suppression (23). In mouse models, $1,25(OH)_2D_3$ enhanced the proliferative capacity of $CD4^+CD25^+$ Treg

(24) and its ability to suppress T helper (Th)2 activity (25). However, there is limited information on the direct effects of $1,25(OH)_2D_3$ on human naturally-occurring Treg. In patients with multiple sclerosis (MS), controversy exists on how serum 25-hydroxyvitamin D_3 levels correlate with peripheral nTreg pool (26;27) though it seems to be implicated in the enhancement of Treg suppressive function (27).

In this study, we assessed the direct effect of $1,25(OH)_2D_3$ on *ex vivo* stimulated human Treg. We show for the first time that human naturally-occurring regulatory T cells express VDR, and consequently that $1,25(OH)_2D_3$ can exert its immunomodulatory effect directly on pre-existing Treg in the absence of APCs. The major effect of $1,25(OH)_2D_3$ on pre-existing Treg is inhibition of proliferation. Other properties associated with its suppressor capacity are left largely unaffected although IL-10 production by Treg was slightly enhanced. Our *in vitro* data on reduced proliferative capacity of Treg is supported by a clinical study whereby decreased numbers of peripheral blood Treg are found during the treatment of vitamin D deficient HIV-infected patients with cholecalciferol.

MATERIALS AND METHODS

Cell isolation

Buffy coats were obtained from healthy donors (Sanquin Blood Bank, Region South East, the Netherlands) with written informed consent on scientific use, according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation with Lymphoprep (Axis-Shield AS, Oslo, Norway) and LeucoSep® (Greiner Bio-One, Frickenhausen, Germany). $CD4^+$ T cells were purified from PBMC by negative selection using monoclonal antibodies (mAbs) directed against CD8 (RPA-T8), CD14 (M5E2), CD16 (3G8), CD19 (4G7), CD33 (P67.6), CD56 (B159) and CD235a (GA-R2(HIR2) (BD-Biosciences, Erembodegem, Belgium) combined with sheep-anti-mouse-Ig coated magnetic beads (Dynal Biotech, Invitrogen ASA, Oslo, Norway). Bead-cell complexes were removed using a magnetic holder. The resultant $CD4^+$ T cell fraction, typically of > 90% purity was incubated with anti-CD25-PE (M-A251, BD Biosciences, NY, USA), anti-CD4-ECD (SFCI12T4D11) and anti-CD27-PC5 (1A4CD27) conjugated antibodies (both from Beckman Coulter Corporation, Miami, USA). $CD4^+CD25^{hi}CD27^+$ regulatory T cells and $CD4^+CD25^{neg}CD27^+$ conventional T cells were isolated from purified $CD4^+$ T cells by high purity flowcytometric cell sorting (Altra Flow Cytometer, Beckman Coulter, Miami, USA). The isolated $CD4^+CD25^{hi}CD27^+$ Treg (routine yield of > 98% purity) and $CD4^+CD25^{neg}CD27^+$

conventional T cells (Tconv) were used immediately after isolation. A phenotypic analysis after isolation established that our target $CD4^+CD25^{hi}CD27^+$ Treg population expressed high level of Foxp3 whereas CD127 expression was lacking. In some experiments, $CD4^+CD25^+$ and $CD4^+CD25^{neg}$ T cells were isolated from the negative isolated $CD4^+$ population by MACS-sorting, using 10 µl anti-CD25 magnetic microbeads for every 10^7 $CD4^+$ T cells (Miltenyi Biotec, Bergisch Gladbach, Germany).

Cell proliferation assay

To study the effect of $1,25(OH)_2D_3$ on cell proliferation, 2.5 x 10^4 Treg or Tconv were stimulated with 5 x 10^3 anti-CD3/anti-CD28 mAb-coated microbeads (Dynal Biotech, Invitrogen ASA, Oslo, Norway) in 200 µl culture medium (RPMI-1640 supplemented with glutamax, 0.02 mM sodium pyruvate, 100 U/ml Penicillin, 100 µg/ml Streptomycin) (all from Gibco, Paisley, UK) and 10% human pooled serum (HPS). Exogenous recombinant human interleukin-2 (rhIL-2) 12.5 U/ml (Proleukine, Chiron, Amsterdam, the Netherlands) was added to the cell culture. The dose-response of $1,25(OH)_2D_3$ (Fluka Biochemika, Sigma-Aldrich, Missouri, USA), dissolved in absolute ethanol, was examined at 1, 10, 100 nM. No solvent effect was apparent in control experiments. Cell cultures were performed in 96-well round bottom plates (Greiner, Frickenhausen, Germany) and incubated in a 37°C, 95% humidity, 5% CO_2 incubator. Cell proliferation was monitored by [^{3}H] Thymidine incorporation using a gas scintillation Counter (Canberra Packard, Matrix 96 Beta-counter, Meriden, USA) on day 3, 4 and 5. The cells were pulsed with 0.5 µCi of [^{3}H] Thymidine (Amersham Biosciences, Piscataway, NJ) during the last 16-18 hours of culture. [^{3}H] Thymidine incorporation is expressed as mean ($\pm$SD) counts per 5 minute of triplicate measurements. Relative inhibition was calculated as: % inhibition= {1- ([^{3}H] Thymidine incorporation co-culture/ [^{3}H] Thymidine incorporation control MLC) x 100%}.

Suppression assay

The suppressive capacity of isolated or cultured Treg was studied in co-culture assays. In brief, graded doses of Treg were added in an increasing ratio to 2.5 x 10^4 responder cells (Tconv) and stimulated with anti-CD3/anti-CD28 mAb-coated microbeads. Cell proliferation was examined by [^{3}H] Thymidine incorporation, as mentioned above, on day 4 of cultures. Relative suppression was calculated as: % suppression= {1- ([^{3}H] Thymidine incorporation co-culture/ [^{3}H] Thymidine incorporation control MLC) x 100%}. To study the suppressive capacity of Treg in the presence of $1,25(OH)_2D_3$, co-cultures were conducted with or without the addition of 100 nM of $1,25(OH)_2D_3$.

Flow cytometry

Cells were phenotypically analyzed by four- and five-color flow cytometry (Coulter Epics XL and Coulter Cytomics FC 500, Beckman Coulter, Fullerton, USA) using Coulter Epics Expo 32 software. Cells were washed with PBS with 0.2% bovine serum albumin (BSA) before being labeled with fluorochrome-conjugated antibodies (mAb). After incubation for 20 minutes at room temperature, in the dark, cells were washed twice to remove unbound antibodies and analyzed. For cell surface staining, the following mAb were used: CD25-PE (M-A251, BD Bioscience, NY, USA), CD4-ECD (SFCI12T4D11) , CD27-PC5 (1A4CD27) (both from Beckman Coulter Corporation, Miami, USA), CD25-PC5 (B1.49.9), CD69-PE (TP1.55.3) (both from Immunotech, Marseille, France) and CD27-PE (M-T271, Dako, Glostrup, Denmark). Appropriate isotype control mAbs were used for marker setting.

Intracellular cytokine staining was performed after 4 hours stimulation with PMA (12.5 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (5 µg/ml; Sigma-Aldrich). Cells were fixed and permeabilized using Fix and Perm reagent (eBioscience, San Diego, USA) according to the manufacturer's recommendations. The following mAb were used for staining: anti-IFNγ-PC7 (4S.B3, eBioscience, San Diego, USA) and anti-IL-2-PE (MQ1-17H12), anti-IL-4-PE (8D4-8), anti-IL-10-PE (JES3-19F1); all from BD Bioscience, NY, USA Intracellular Foxp3 expression was analyzed after fixation and permeabilization using anti-human FoxP3 (FCH101) mAb, FITC or PE labeled; all from eBioscience, San Diego, USA). Similarly, Ki-67 expression was examined by intracellular staining using anti-human Ki-67-FITC mAb (B56, BD Bioscience, NY, USA)

Vitamin D receptor expression on T cells was analyzed by an indirect staining method. After fixation and permeabilization of cell sample, cells were incubated with anti-human VDR mAb (NR1I1, R&D Systems, Minneapolis, USA) at 4°C for 30 minutes. Cells were then washed twice with permeabilization buffer (eBioscience, San Diego, USA) followed by incubation with goat-anti-mouse IgG-FITC (Dako, Glostrup, Denmark) in the dark, at 4°C for 30 minutes and analyzed by flow cytometry. Appropriate isotype (IgG_{2A} mAb) and conjugate control staining were included.

CFSE-based cell division analysis

Cell division was studied by CFSE dilution analysis. To this end, high purity sorted cells, Treg (0.5–2 x 10^6) and Tconv (10 x 10^6) were labeled with 0.25–0.5 µM and 4 µM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) respectively. The cells were subsequently activated with anti-CD3/anti-CD28 mAb-coated microbeads with or without the

addition of 100 nM $1,25(OH)_2D_3$. Cell division accompanied by CFSE dilution was analyzed by flow cytometry on days 1, 2 and 5.

Stimulation assay to study T cell anergy

To study T cell anergy, 2.5 x 10^4 sorted Treg and 2.5 x 10^4 Tconv were stimulated with anti-CD3/anti-CD28 mAb-coated microbeads, 25 U/ml rhIL-2 and 10 ng/ml IL-15 (Biosource International, Camarillo, CA); with or without $1,25(OH)_2D_3$. After expansion, the cells were harvested on day 7, washed and rested for 2 days in culture medium containing 5% HPS. Next secondary cell cultures were performed whereby cells were stimulated with anti-CD3/anti-CD28 mAb-coated microbeads with and without the addition of 12.5 U/ml IL-2. Cell proliferation was examined by [^{3}H] Thymidine incorporation, as described above, after 2 days.

***In vivo* study**

An observational study was done among vitamin D deficient HIV-1 seropositive patients visiting the Radboud University Nijmegen Medical Centre, The Netherlands. Vitamin D deficiency was defined as $25(OH)D_3$ levels below 25 or 35 nmol/L, depending on the season. We prospectively studied the effect of cholecalciferol supplementation on circulating Treg numbers by flow cytometry. The subjects were treated with a daily dose of 2000 IU cholecalciferol (vitamin D_3) during the first 12 weeks and thereafter for at least 48 weeks with a dose of 1000 IU daily. At baseline and after 24 and 48 weeks serum $25(OH)D_3$, 1,25-dihydroxyvitamin D_3, parathyroid hormone (PTH) levels and circulating Treg were determined. This study was approved by the local ethics committee and written informed consent was given by all participating subjects

Twenty subjects were included but two subjects discontinued cholecalciferol supplementation after 24 weeks for non-medical reasons. No adverse effects were reported or observed during the supplementation.

$25(OH)D_3$, $1,25(OH)_2D_3$ and parathyroid hormone determination

Serum $25(OH)D_3$ was measured by high pressure liquid chromatography (HLPC) with UV detection, after prior extraction on small SepPak columns. Tritiated 25-hydroxyvitamin D_3, collected from the HPLC system during passage of the UV peak, was used to correct for procedural losses. Serum $1,25(OH)_2D_3$ was measured by radioreceptor assay (RRA) with prior extraction and chromatographic purification with correction for recovery. For PTH assays the ELSA-PTH assay by CIS BIO was used at first. This method was switched into the intact PTH assay performed by the Abbott Architect analyzer, when the CIS BIO assay

was no longer available. The Abbott Architect assay was recalibrated on the CIS BIO assay to give identical measurement results.

Statistical analysis

Statistical analysis was performed using the Statistical Product and Services Solutions (SPSS) package version 16.0. The Wilcoxon rank-sum test was used to compare differences between groups (unless otherwise stated). The level of significance was set at $P < 0.05$.

RESULTS

Human regulatory T cells express VDR

$1,25(OH)_2D_3$ can only exert its effect through binding to its receptor. Although the expression of VDR by T lymphocytes has been reported (2;3), no information is available on the expression of VDR by Treg. Using flow cytometric analysis, we showed that both freshly isolated $CD4^+CD25^{hi}$ Treg as well as $CD4^+CD25^{neg}$ conventional T cells express VDR (Figure 1A). VDR expression was increased upon activation with anti-CD3/anti-CD28 mAb-coated microbeads in Treg (Figure 1B) as well as Tconv (Figure 1C). The highest levels of VDR expression by Treg and T conv were found were found on day 2 of culture.

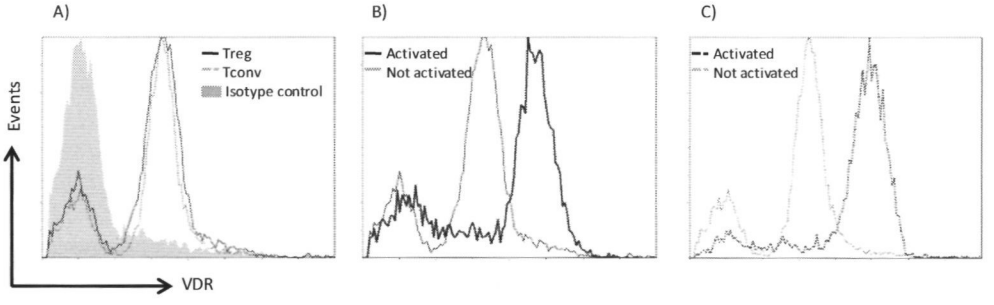

Figure 1. Flow cytometry of VDR expression by Treg and Tconv

A) Histogram overlay of VDR expression in Treg and Tconv with isotype staining. Histograms showing VDR expression (X-axis) in anti-CD3/anti-CD28 mAb-coated microbeads stimulated vs un-stimulated B) Treg and C) Tconv on day 2. Data are representative for 3 independent experiments performed with cells obtained from different donors.

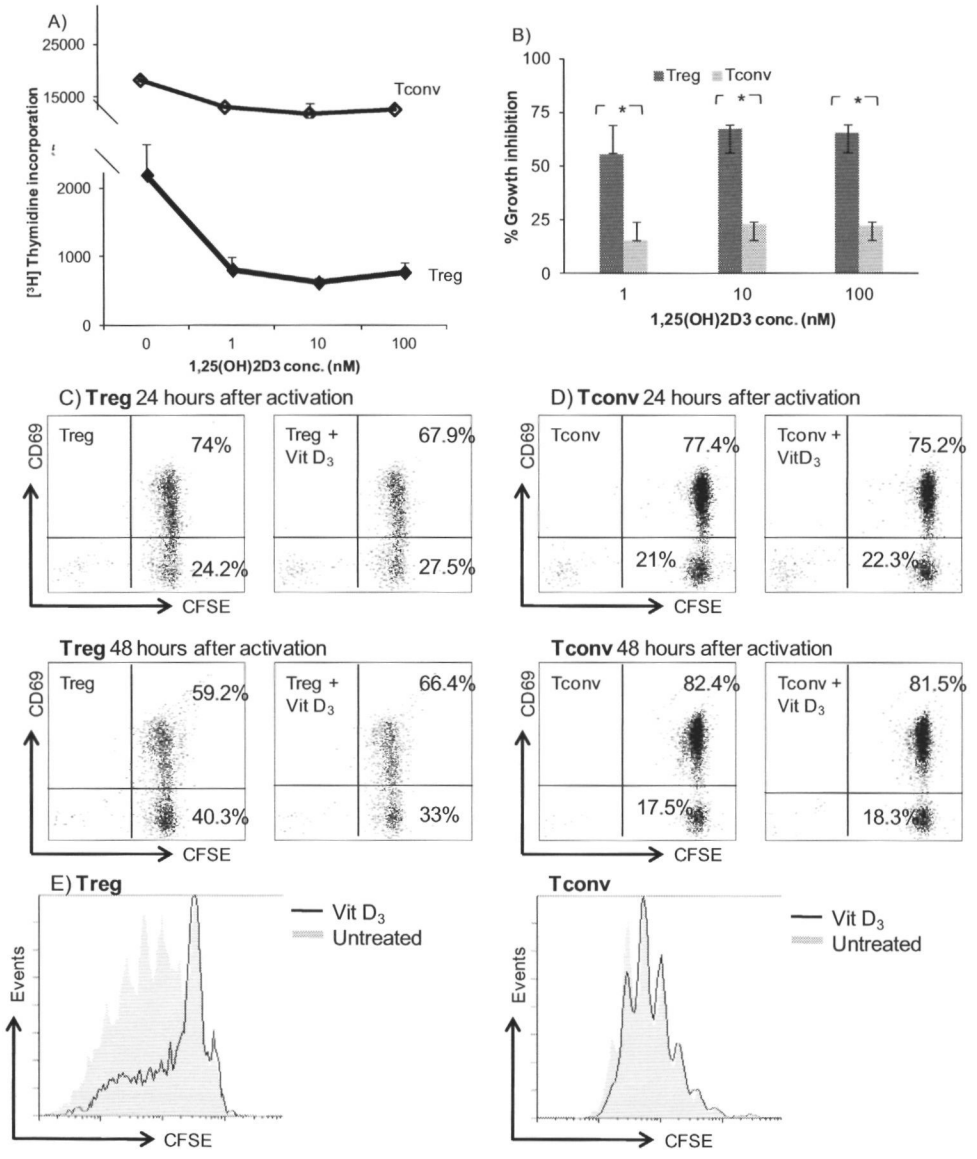

Figure 2. 1,25(OH)$_2$D$_3$ inhibits Treg and Tconv proliferation

A) The effect of graded doses of 1,25(OH)$_2$D$_3$ on Treg or Tconv proliferation, stimulated with anti-CD3/anti-CD28 mAb-coated microbeads and in the presence of IL-2. Cell proliferation as assayed by [^{3}H] Thymidine incorporation at day 4 of culture and expressed as mean ± SD. Data are representative for 5 independent experiments. B) Percentage growth inhibition of Treg and Tconv upon treatment with 1,25(OH)$_2$D$_3$ at 1, 10 and 100 nM was calculated. Data are the cumulative results of 5 independent experiments using cells isolated by high purity flowcytometric cell sorting. * $P < 0.05$ when comparing the degree of growth inhibition between Treg and Tconv cells. Effect of 1,25(OH)$_2$D$_3$ (100 nM) on CD69 expression (Y-axis), in C) Treg and D) Tconv labeled with CFSE (X-axis), after 24 or 48 hours post-activation. E) Cell division of Treg and Tconv as analyzed by CFSE dilution (X-axis) on day 5 with and without addition of 100 nM 1,25(OH)$_2$D$_3$. Data are representative for 3 independent

experiments performed with cells isolated by high purity flowcytometric cell sorting, obtained from different donors.

$1,25(OH)_2D_3$ inhibits proliferation, but allows activation of Treg

Having established that Treg express VDR, we assessed whether vitamin D_3 can affect Treg proliferation in an APC-free system. Treg were stimulated with anti-CD3/anti-CD28 mAb-coated microbeads, in the presence of exogenously added rhIL-2 and 1, 10 or 100 nM $1,25(OH)_2D_3$. Treg proliferation as analyzed by [^{3}H] Thymidine incorporation was clearly inhibited in a dose dependent fashion (Figure 2A). Even at a low concentration of 1 nM $1,25(OH)_2D_3$ there was a marked decrease in cell proliferation. Interestingly, we observed a significant difference in the inhibition of cell division between Treg and Tconv (Figure 2B and 2E), indicating that Treg proliferation was more susceptible to the suppressive effect of vitamin D_3. This difference between Treg and Tconv was consistently observed through all the concentrations of $1,25(OH)_2D_3$ employed (Figure 2B).

Since the addition of vitamin D_3 significantly inhibited Treg proliferation, we wondered whether this compound also interfere with early T cell activation. To analyze this, we looked at cell activation status by measuring the expression of CD69, an inducible cell surface glycoprotein acquired very early during T cell activation. We found that treatment with $1,25(OH)_2D_3$ did not cause any difference in the expression of CD69 in Treg (Figure 2C) or Tconv (Figure 2D) within the first 24 to 48 hours post activation. Although $1,25(OH)_2D_3$ allowed activation of Treg, cell cycle progression was inhibited (Figure 2E).

Vitamin D_3 treatment preserves Treg suppressor function, anergic phenotype and Foxp3 expression

Treg are characterized by its suppressor function, anergic phenotype and constitutively high expression of the transcription factor Foxp3. Here, we assessed the influence of vitamin D_3 on the aforementioned Treg characteristics. To examine whether $1,25(OH)_2D_3$ treatment interferes with the suppressor activity of Treg, freshly isolated Treg were stimulated with anti-CD3/anti-CD28 mAb-coated microbeads in the presence of $1,25(OH)_2D_3$ and cultured for 7 days. Thereafter, the cells were allowed to recuperate and tested for their suppressor function in a co-culture assay. After this 'pre-treatment' with $1,25(OH)_2D_3$, Treg kept their suppressive capacity (Figure 3A). Additionally, we demonstrated that the suppressive activity

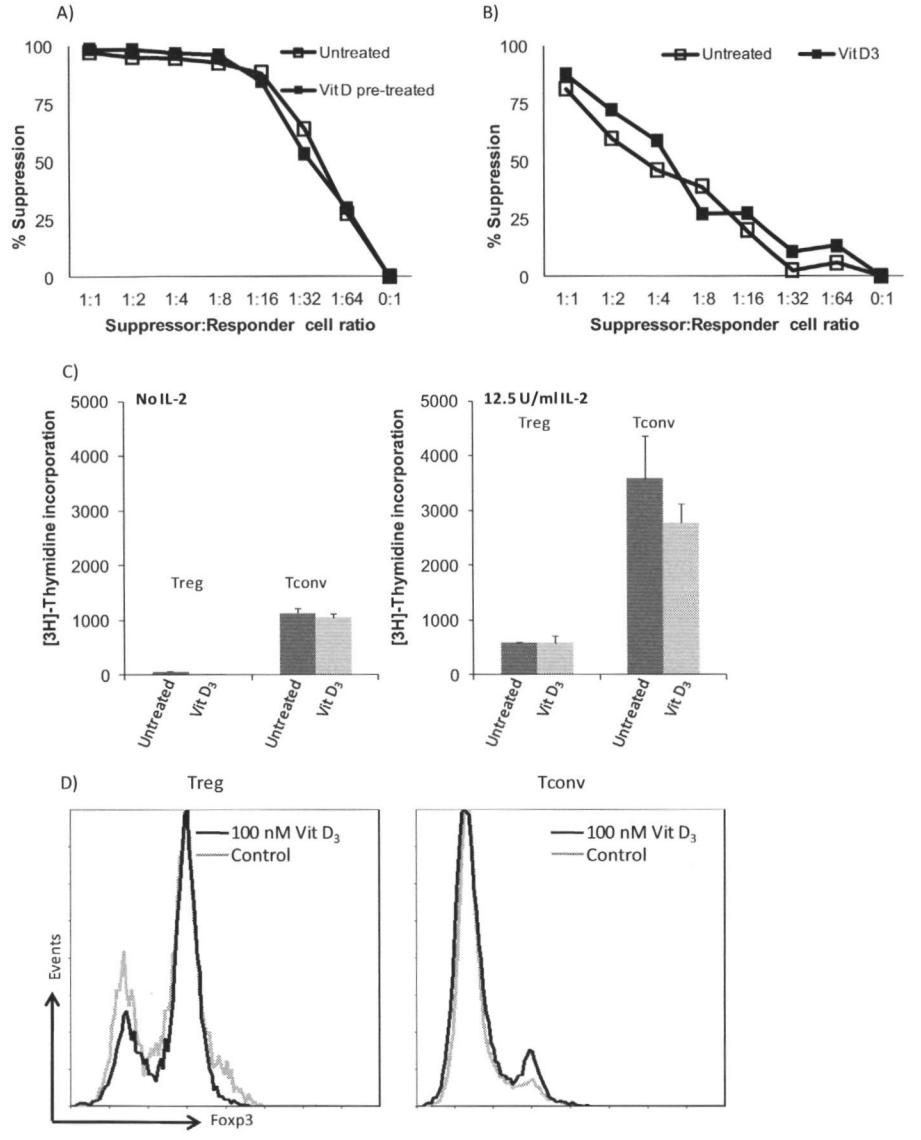

Figure 3. Effect of $1,25(OH)_2D_3$ on Treg suppressive function and phenotypic characteristics

A) Treg were expanded with anti-CD3/anti-CD28 mAb-coated microbeads with or without 100 nM $1,25(OH)_2D_3$ for 7 days. The cells were harvested and rested for 2 days. Co-culture suppression assays were conducted to analyze the suppressor potential of these $1,25(OH)_2D_3$-pretreated cells. Cell proliferation was determined by [^{3}H] Thymidine incorporation at day 4. Results are expressed as percentage suppression= {1- ([^{3}H] Thymidine incorporation co-culture/ [^{3}H] Thymidine incorporation control MLC) x 100%} (Y-axis). B) Suppression assay performed in the absence or presence of 100 nM $1,25(OH)_2D_3$. Percentage suppression (Y-axis) was measured and calculated as mentioned above. C) Treg and Tconv were expanded with anti-CD3/anti-CD28 mAb-coated microbeads with or without 100 nM $1,25(OH)_2D_3$ for 7 days. The cells were harvested and rested for 2 days. Proliferative capacity of expanded cells upon restimulation with anti-CD3/anti-CD28 mAb-coated microbeads was determined in the absence or presence of IL-2 by [^{3}H] Thymidine incorporation (Y-axis). D)

Representative histogram overlay depicting the expression of Foxp3 (Y-axis) in Treg and Tconv on day 8, as measured by flow cytometry. Data are representative for 5 or 6 independent experiments performed with cells isolated by high purity flowcytometric cell sorting, obtained from different donors. *P < 0.05 as compared to respective cell culture without the addition of $1,25(OH)_2D_3$.

of Treg was not affected when a high concentration of $1,25(OH)_2D_3$ was present during the co-culture assay (Figure 3B).

Another classical characteristic of Treg is that they exhibit anergic behavior *in vitro*. Classically, T cell anergy is defined as a low proliferative capacity upon stimulation with antigen only, which can be (partially) reversed by addition of exogenous IL-2. This feature was examined in restimulation assays. We found that independent of the provision of $1,25(OH)_2D_3$ to the primary cell culture, Treg retained their anergic state (Figure 3C). In contrast to Tconv, Treg did not proliferate in the secondary culture upon stimulation in the absence of IL-2 while the addition of IL-2 to the secondary culture restored their proliferative capacity.

Foxp3 expression was examined by flow cytometry 8 days after Treg stimulation with anti-CD3/anti-CD28 mAb-coated microbeads in the presence of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ did not affect Foxp3 expression in Treg. Neither the level of Foxp3 expression per cell as indicated by the mean fluorescence intensity (MFI) nor the percentage of cells expressing Foxp3 was affected (Figure 3D).

Vitamin D_3 increases the percentage of Treg producing IL-10 in an APC-free system

Having established that $1,25(OH)_2D_3$ diminished the proliferative capacity of Treg but spared their suppressive function, we evaluated the influence of $1,25(OH)_2D_3$ on cytokine production (IL-2, IFNγ, IL-4 and IL-10) in isolated Treg by intracellular flow cytometry. With the addition of 100 nM $1,25(OH)_2D_3$, we found a statistically significant increase in the percentage of Treg producing IL-10 upon stimulation with anti-CD3/anti-CD28 mAb-coated microbeads, in an APC-free system (Figure 4A). IL-2 and IFNγ production in both Treg and Tconv was unaffected by $1,25(OH)_2D_3$ (Figure 4B).

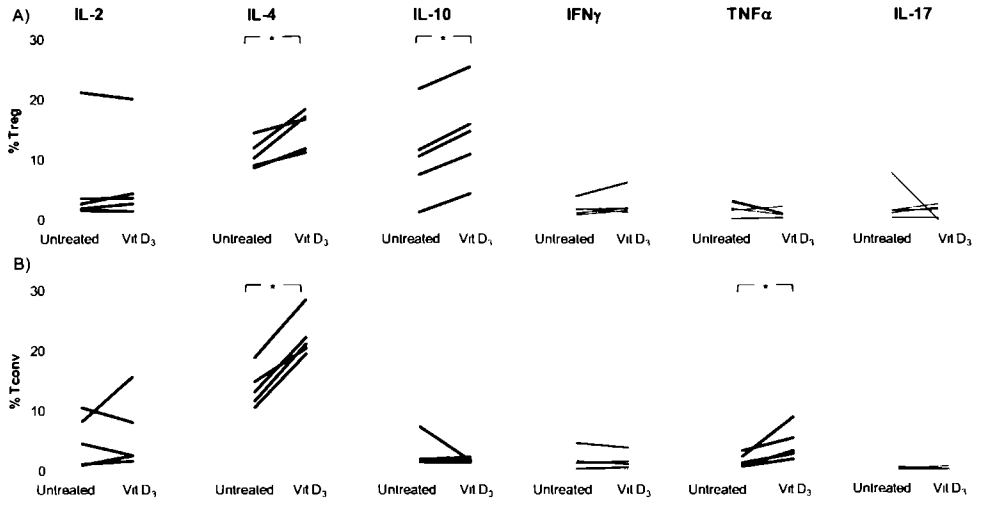

Figure 4. Effect of $1,25(OH)_2D_3$ on cytokine production

Intracellular staining for cytokines in A) Treg and B) Tconv. Treg and Tconv were stimulated with anti-CD3/anti-CD28 mAb-coated microbeads with or without the addition of 100 nM $1,25(OH)_2D_3$. At day 8 of cultures, the cells were stimulated with PMA plus ionomycin in the presence of brefeldin A and intracellularly stained with antibodies directed against the cytokine as shown above. Data are representative for 5 independent experiments performed with cells isolated by high purity flowcytometric cell sorting, obtained from different donors. *P < 0.05 as compared to respective cell culture without the addition of $1,25(OH)_2D_3$.

In patients supplemented with cholecalciferol, the proliferative capacity of Treg was reduced and lower numbers of Treg were found

Having established inhibitory effects of $1,25(OH)_2D_3$ on Treg proliferation *in vitro*, we looked for evidence of this feature *in vivo*. To this end, we studied the effect of cholecalciferol (vitamin D_3) supplementation on Treg cell division and Treg numbers in HIV-1 seropositive patients, who are known to have low serum $25(OH)D_3$ and $1,25(OH)_2D_3$ levels (13). The subjects were supplemented with an oral daily dose of 2000 IU cholecalciferol during the first 12 weeks and thereafter for at least 48 weeks with a dose of 1000 IU daily. At baseline and after 24 and 48 weeks after supplementation, we determined the numbers and proliferative capacity of circulating $CD4^+CD25^{hi}$ Treg and the serum levels of $25(OH)D_3$, $1,25(OH)_2D_3$, and parathyroid hormone .

Upon supplementation of cholecalciferol, the serum $25(OH)D_3$ concentration significantly increased after 24 and 48 weeks as compared to baseline levels (Table 1). Also, $1,25(OH)_2D_3$ levels significantly increased after 24 weeks of supplementation, but were not significantly different from baseline at 48 weeks (Table 1, Figure 5A). As expected,

parathyroid hormone levels had an inverse relation to $1,25(OH)_2D_3$ levels. Interestingly, the percentages and absolute numbers of $CD4^+CD25^{hi}CD127^-Foxp3^+$ Treg were significantly lower after 24 weeks of supplementation, but recovered at 48 weeks when the dosage of cholecalciferol was lowered at week 12 (Figure 5B). This suggests a direct and inverse relationship between the levels of $1,25(OH)_2D_3$ and the number of circulating Treg. Next, we analyzed the *ex vivo* proliferative capacity of Treg in supplemented patients by measuring the expression of the proliferation marker Ki-67 in $CD4^+Foxp3^+$ expressing Treg using flow cytometry (Figure 5C). At first, we compared the proliferation status of Treg in 8 subjects after 24 and 48 weeks supplementation of cholecalciferol, which corresponded to reduced $1,25(OH)_2D_3$ levels at week 48 as compared to week 24. We found an increased expression of Ki-67 in the Treg population (Figure 5D), but not in $CD4^+$ Tconv (i.e. $Foxp3^-$ cells) (Figure 5E) at week 48 as compared to week 24. Together, these findings show that increased $1,25(OH)_2D_3$ levels concur with reduced proliferative capacity of Treg *in vivo*.

	Mdn (IQR)	Mdn (IQR)	Mdn (IQR)	p-value	p-value
Week	**0**	**24**	**48**	**0-24**	**0-48**
25-hydroxyvitamin D_3 (nmol/L)	26.4 (19.0-29.1)	98.5 (68.3-103.5)	79.8 (62.9-98.4)	<.001	<.001
1,25-dihydroxyvitamin D_3 (pmol/L)	142 (106-167)	186 (108-215)	132 (99-144)	0.005	0.339
Parathyroid hormone (pmol/L)	4.70 (4.0-6.1)	4.2 (2.9-4.8)	5.0 (3.4-6.4)	0.021	0.398
Treg number (cells/L)	19 (12-28)	9 (6-14)	24 (11-38)	0.005	0.129
Treg in CD4 population	4.3 (2.3-5.7)	2.0 (1.5-3.1)	5.6 (2.4-10.1)	0.001	0.068

Table 1. Baseline data and week 24 and 48 data after cholecalciferol supplementation in 25-hydroxyvitamin D_3 deficient HIV-1 positive subjects

DISCUSSION

In this study, we showed a direct effect of $1,25(OH)_2D_3$ on nTreg independent of APC. $1,25(OH)_2D_3$ dose-dependently inhibited Treg proliferation *in vitro* and high levels of serum $25(OH)D_3$ and $1,25(OH)_2D_3$ concur with low frequency of Treg *in vivo*. Apart from its inhibitory effect on Treg proliferation, $1,25(OH)_2D_3$ had no influence on either the expression

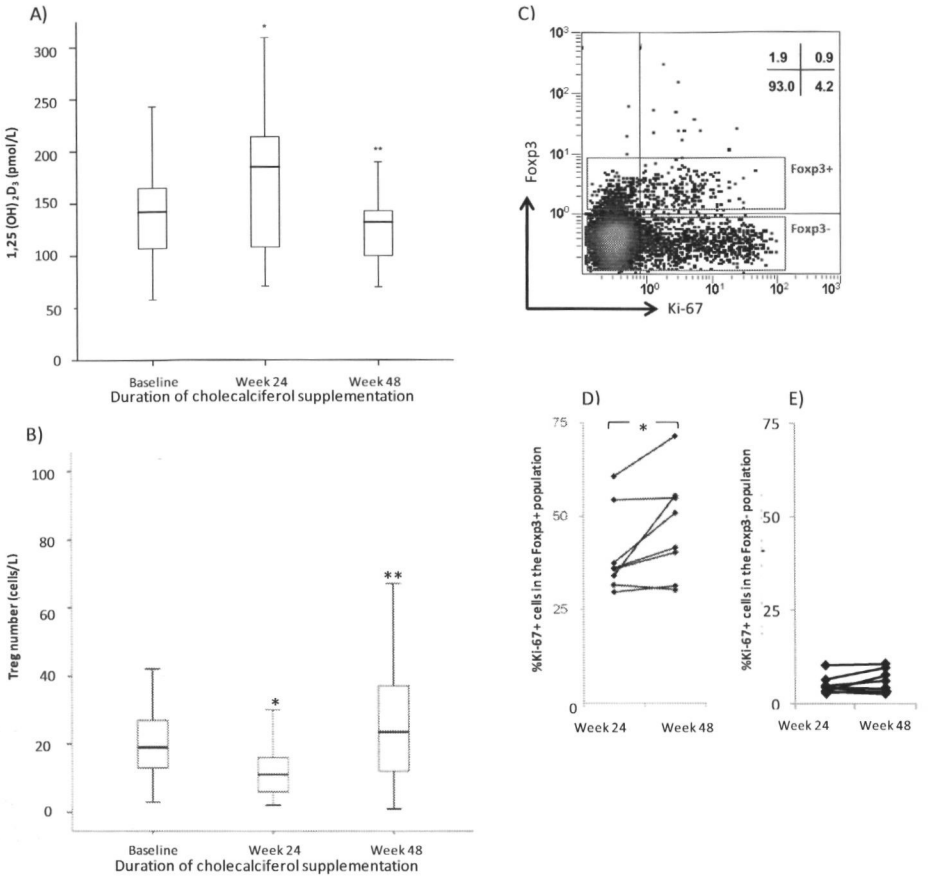

Figure 5. Effect of cholecalciferol supplementation in vitamin D_3 deficient HIV-infected patients

A) Changes in serum $1,25(OH)_2D_3$ concentrations and B) Treg numbers over time. *P < 0.05 as compared to baseline, **P < 0.05 as compared to week 24. The subjects were treated with a daily dose of 2000 IU cholecalciferol during the first 12 weeks and thereafter for at least 48 weeks with a dose of 1000 IU daily. C) Representative flow cytometry dotplot showing intracellular Ki-67 (X-axis) and Foxp3 (Y-axis) staining on $CD4^+$ gated T cells. Cumulative data showing the percentages of Ki-67 expressing cells within the D) $CD4^+Foxp3^+$ and E) $CD4^+Foxp3^-$ population at week 24 and week 48 in 8 subjects, where serum $25(OH)D_3$ levels at week 48 was reduced as compared to week 24. *P < 0.05 as compared to week 24.

of Foxp3 in Treg or on the anergic characteristic and suppressive function *in vitro*, and even induced a slight increase in IL-10 production by Treg in an APC-free system. Also, we showed an inverse relationship between *in vivo* $1,25(OH)_2D_3$ levels and Treg numbers in HIV-infected patients supplemented with cholecalciferol. So, whereas vitamin D_3 may de novo induce Treg via the induction of tolerogenic DC (28;29) its direct effect on pre-existing Treg appears containment of the population size.

The active form of vitamin D, 1,25-dihydroxyvitamin D_3, exerts its biological effect through VDR leading to modified gene expression. The vitamin D receptor is part of the steroid hormone nuclear receptor family that are expressed by various cell types including mononuclear cells. However, to our knowledge, no information is available regarding the expression of VDR by Treg. In this study, we demonstrated that Treg, like Tconv, do express VDR, and that the expression was up regulated upon T cell receptor (TCR) stimulation. This implies that $1,25(OH)_2D_3$ may exert its immunomodulatory effects directly on Treg as we show here for cytokine production and proliferation arrest. The interplay between VDR and TCR signaling has not been widely studied. Although not specific to Treg, one possible mechanism involves TCR signaling via protein kinase p38 pathway which has been shown to induce up-regulation of vitamin D receptor and phospholipase C (PLC)-gamma1 in $CD4^+$ and $CD8^+$ T cells (30). In addition, induction of PLC-gamma1 was found to be dependent on vitamin D and expression of the VDR and was vital for subsequent classical TCR signaling and T cell activation.

Here, we showed clearly that $1,25(OH)_2D_3$ inhibit the proliferation of human Treg freshly isolated from peripheral blood. Interestingly, we observed that in comparison to Tconv cells, $1,25(OH)_2D_3$ had a more pronounced repressive effect on Treg with regards to proliferation. Despite the fact that $1,25(OH)_2D_3$ inhibited proliferation of Treg *in vitro*, it did not affect the number of Tregs that became activated upon stimulation with anti-CD3/anti-CD28 mAb-coated microbeads, as indicated by the expression of CD69, nor did it induce cell death in Treg (data not shown).

Our finding adds to the already described immunomodulatory effects of $1,25(OH)_2D_3$ on APC and conventional T lymphocytes. In the presence of APC, $1,25(OH)_2D_3$ can induce a regulatory phenotype by influencing their maturation (22;31), hereby facilitating the induction of regulatory cells via modulation of the APC. In pre-existing nTreg we showed that $1,25(OH)_2D_3$ inhibited its proliferation, but spared the suppressive phenotype and to some extent increased the number of IL-10 producing cells, notably in an APC-free system employed by the present study. With regard to conventional T cells, it has been shown that $1,25(OH)_2D_3$ and its analogues have the capacity to inhibit T cell proliferation (32-34) and to modulate cytokine production (35;36) when activated with mitogens. Secretion of IFNγ and IL-17 was inhibited (23), and IL-4 and IL-10 production was enhanced (23;37-39) thereby favouring a bias towards Th2 differentiation.

In vivo we found suggestive evidence that cholecalciferol supplementation affect Treg numbers and proliferation. Increasing serum $25(OH)D_3$ and $1,25(OH)_2D_3$ levels were paralleled by a drop in peripheral Treg numbers, while a subsequent reduction in $25(OH)D_3$ levels resulted in recovery of Treg numbers. This variation in $25(OH)D_3$ and $1,25(OH)_2D_3$ levels concurred with changes in the number of dividing $Foxp3^+$ cells, suggesting that regulation of Treg cell division was the underlying effect of cholecalciferol supplementation *in vivo*. Importantly, resting Tregs have recently been identified as a reservoir for HIV replication. *In vitro* activation of these cells led to a detectable viral production (40). The authors suggested that resting Tregs could release the virus when their anergy status is disturbed. We showed that vitamin D_3 can inhibit Treg proliferation *in vivo* while *in vitro*, it preserved anergy. Therefore, $1,25(OH)_2D_3$ could be possibly beneficial in diminishing the latent viral pool. Having said that, this postulation requires further verification.

In conclusion, the present study demonstrated that nTreg express VDR which can be up-regulated upon activation. The immediate effect of $1,25(OH)_2D_3$ on nTreg is growth inhibition without interference on its suppressive capacity, anergy status or the Foxp3 expression. Also, *in vitro* $1,25(OH)_2D_3$ skewed cytokine secretion in nTreg towards IL-10 producing cells in the absence of APC. As *in vivo* proof of principle that vitamin D_3 inhibits Treg proliferation, we demonstrated that cholecalciferol supplementation in HIV-infected subjects restored $1,25(OH)_2D_3$ and $25(OH)D_3$ levels resulting in a decreased Treg numbers.

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High Dose Vitamin D_3 Supplementation is a Requisite for Modulation of Skin-homing Markers on Regulatory T Cells in HIV-infected Patients

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ABSTRACT

Vitamin D_3 is known to have an effect on the immune function. We investigated the immunomodulatory capability of vitamin D_3 in human immunodeficiency virus (HIV)-infected patients and studied the expression of chemokine receptors on regulatory T cell (Treg). Vitamin D_3-deficient HIV-1 seropositive subjects were treated with cholecalciferol (vitamin D_3) at a daily dose of 800 IU for 3 months (n = 9) or a weekly dose of 25000 IU for 2 months (n = 7). Peripheral blood mononuclear cells (PBMC) were isolated and analyzed for skin-homing (CCR4 and CCR10) and gut-homing (CCR9 and integrin $\alpha_4\beta_7$) marker expression on Treg, by flow cytometry. Serum 25(OH)D_3 and PTH levels were determined at baseline and after the treatment period. Weekly doses of 25000 IU cholecalciferol effectively achieved the optimal target serum 25(OH)D_3 concentration of >75 nmol/L in HIV-infected patients. High dose cholecalciferol supplementation differentially influenced skin-homing markers on Treg with an increased level of CCR10 expression and while a reduction in CCR4 expression level was observed together with a lower percentage of Treg expressing CCR4. For both dosing regimens, there were no significant differences in the expression of gut-homing markers, CCR9 and integrin $\alpha_4\beta_7$. In conclusion, high dose vitamin D_3 supplementation is needed to reverse vitamin D_3 deficiency in HIV-infected individuals and this result in modulation of skin-homing markers but not gut-homing markers expression on Treg. At a standard dose of 800 IU/day, vitamin D_3 is not effectively in achieving an optimal 25(OH)D_3 concentration in patients with an underlying T cell dysfunction and is unable to exert any immunomodulatory effects.

INTRODUCTION

Vitamin D_3 deficiency has also been identified as a highly prevalent entity among human immunodeficiency virus (HIV)-infected patients (1). The deficiency can be seen in both treated and untreated cohorts as well as HIV-infected patients compared to uninfected controls (2;3). Vitamin D_3 has expanded its role beyond calcium and bone metabolism. The association of vitamin D_3 deficiency with autoimmune conditions and infectious diseases provide the impetus to evaluate the impact of vitamin D_3 supplementation on HIV disease and antiretroviral therapy. A well recognized effect of vitamin D_3 has been its capacity to modulate T cell growth and function, and antigen presenting cell differentiation (4;5). It has been demonstrated that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) inhibit T cell proliferation through suppression of interleukin (IL)-2 production (6;7). $1,25(OH)_2D_3$ has also been shown to affect regulatory T cell (Treg) function and proliferation (8;9). Regulatory T cells, as characterized by the phenotype $CD4^+CD25^{hi}$ and the transcription factor, forkhead box protein 3 (Foxp3), are crucial in the regulation of T cell homeostasis through its suppressive role on $CD4^+CD25^-$ T cell response (10). This regulatory function of Treg is known to be hampered in autoimmunity, allergy and infectious diseases, indicating that these cells play a crucial role in immune-mediated pathology. However, little is known of the effects of vitamin D_3 on T cell or Treg immunity in HIV-infected patients. This is so due to the limited number of studies carried out using vitamin D_3 supplementation alone (11;12) or performed to assess its effects other than bone health in this group of patients.

Human immunodeficiency virus-1 infection is characterized by progressive $CD4^+$ T cell depletion and immune dysfunction. Regulatory T cells are potential targets of HIV given that they express CD4 (13). HIV entry into target cells also requires cellular expression of the chemokine receptors CCR5 and CXCR4 in conjunction with CD4 (14). The role of Treg in regulating T cell activation during immune responses to pathogens such as chronic viral infections is a subject of great interest. Their effects can be beneficial or detrimental hinging on the balance between attenuating HIV-induced immune hyperactivation and mounting an immune response to HIV and mucosal pathogens. Chronic HIV infection also seems to alter the distribution of Tregs resulting in an apparent increased proportion in the peripheral lymph nodes and mucosal lymphoid tissues (15-18). Various chemokine receptors such as CCR4, CCR9, CCR10, CD62L and integrin $\alpha_4\beta_7$ are known to dictate the homing capacity of T cells as well as Tregs (19;20). Using $CD4^+CD25^+$ Tregs isolated from healthy subjects, Ji et al demonstrated that HIV-1 binds to Treg leading to an up-regulation of CD62L and integrin

$\alpha_4\beta_7$ expression (18). As a result, Tregs migrate to and accumulate in the peripheral lymph nodes and mucosal lymphoid tissues.

Vitamin D_3 has also been shown to affect the homing capacity of the peripheral $CD4^+$ T cell population. Sigmundsdottir et al reported that $1,25(OH)_2D_3$ induced the expression of skin-homing chemokine receptors, CCR10 on $CD4^+$ T cells *in vitro,* at the expense of gut-homing markers, CCR9 and integrin $\alpha_4\beta_7$ (21). Given that both vitamin D_3 (21) and HIV (15;16;18) can modulate the expression of chemokine receptors on Treg, it brings to mind whether the homing capability of Treg can be augmented by vitamin D_3 during HIV infection. We investigate how cholecalciferol (vitamin D_3) in two different dosing regimen, 800 IU daily versus 25000 IU weekly, can impact Treg homeostasis in HIV-infected individuals and evaluate the expression of skin- and gut-homing markers *in vivo* and *in vitro*.

MATERIALS AND METHODS

Patients

From April 2007 to August 2009, we conducted a pilot study in vitamin D_3-deficient HIV-infected patients visiting the Radboud University Nijmegen Medical Centre, The Netherlands. Patients included were HIV-1 seropositive, vitamin D_3 deficient and above 18 years old. Vitamin D_3 deficiency was defined as $25(OH)D_3$ levels below 35 nmol/L (April to September) and below 25 nmol/L (October to March). The low-dose study cohort (n = 9) received a daily oral dose of 800 IU cholecalciferol for 3 months. The high-dose cohort (n = 7) received a weekly oral dose of 25000 IU cholecalciferol for 2 months. In the control group (n = 7) recruited from February to May 2009 and followed through until May to August 2009, no supplementation was administered. At baseline and after the treatment period, blood samples were taken for peripheral blood mononuclear cells (PBMC) isolation preceding flow cytometry analysis and serum vitamin D_3 levels. The study was approved by the Ethical Committee on Human Experimentation of the Radboud University Nijmegen. A written consent was obtained from all participants to the study. PBMC were isolated as described below and subsequently used for flow cytometry analysis.

In vitro study

Buffy coats were obtained from healthy donors (Sanquin Blood Bank, Region South East, the Netherlands). Informed consent was obtained according to the Declaration of Helsinki. PBMC were isolated by density centrifugation with Lymphoprep (Axis-Shield AS, Oslo,

Norway) and LeucoSep® (Greiner Bio-One, Frickenhausen, Germany). $CD4^+$ T cells were purified from PBMC by negative selection using monoclonal antibodies (mAbs) directed against CD8 (RPA-T8), CD14 (M5E2), CD16 (3G8), CD19 (4G7), CD33 (P67.6), CD56 (B159) and CD235a (GA-R2(HIR2) (BD-Biosciences, Erembodegem, Belgium) combined with sheep-anti-mouse-Ig coated magnetic beads (Dynal Biotech, Invitrogen ASA, Oslo, Norway). The resultant $CD4^+$ T cell fraction, typically of > 90% purity was incubated with anti-CD25-PE (M-A251, BD Biosciences, NY, USA), anti-CD4-ECD (SFCI12T4D11) and anti-CD27-PC5 (1A4CD27) conjugated antibodies (both from Beckman Coulter Corporation, Miami, USA). $CD4^+CD25^{hi}CD27^+$ regulatory T cells (Treg) were isolated from purified $CD4^+$ T cells by high purity flowcytometric cell sorting (Altra Flow Cytometer, Beckman Coulter, Miami, USA). The isolated $CD4^+CD25^{hi}CD27^+$ Treg routine yield of > 95% purity were stimulated with anti-CD3/anti-CD28 mAb-coated T cell expander beads (Dynal Biotech, Invitrogen ASA, Oslo, Norway) in 200 µl culture medium (RPMI-1640 supplemented with glutamax, 0.02 mM sodium pyruvate, 100 U/ml Penicillin, 100 µg/ml Streptomycin) (all from Gibco, Paisley, UK) and 10% human pooled serum (HPS). Exogenous recombinant human interleukin-2 (rhIL-2) 12.5 U/ml (Proleukine, Chiron, Amsterdam, the Netherlands) was added to the cell culture. 1,25-dihydroxyvitamin D_3 (Fluka Biochemika, Sigma-Aldrich, Missouri, USA) was dissolved in absolute ethanol and made up to a concentration of 100 nM in each well. No solvent effect was apparent in control experiments. Cell cultures were performed in 96-well round bottom plates (Greiner, Frickenhausen, Germany) and incubated for 3-4 days in a 37°C, 95% humidity, 5% CO_2 incubator.

Flow cytometry

Cells were phenotypically analyzed by five-color flow cytometry (Coulter Cytomics FC 500, Beckman Coulter, Fullerton, USA) using Coulter Epics Expo 32 software. Cells were washed with PBS with 0.2% bovine serum albumin (BSA) before being labeled with fluorochrome-conjugated antibodies (mAb). After incubation for 20 minutes at room temperature, in the dark, cells were washed twice to remove unbound antibodies and analyzed. For cell surface staining, the following mAb were used: CD127 PC5- or PC7-labeled (RDR5) (both from eBioscience); CD25-PE (M-A251), CD25-APC (2A3), CD49d-PE (9F10), CCR4-PC7 (1G1), (all from BD Biosciences); CD4 ECD- or PC7-labeled (SFCI12T4D11), CD4-PC5 (13B8.2), CD27-PC5 (1A4CD27), (all from Beckman Coulter Corporation); CCR9-PE (112509), CCR10-PE (314305) (both from R&D Systems) and CD27-FITC (M-T271; Dako). Appropriate isotype control mAbs were used for marker setting.

25(OH)D_3 and parathyroid hormone levels

Serum 25(OH)D_3 was measured by high pressure liquid chromatography (HLPC) with UV detection, after prior extraction on small SepPak columns as previously described(2). Tritiated 25(OH)D_3, collected from the HPLC system during passage of the UV peak, was used to correct for procedural losses. The within run precision was 2.6% at 69 nmol/l and between run precision was 6.2% at 69 nmol/L. For parathyroid hormone (PTH) assays the ELSA-PTH assay by CIS BIO was used at first. This method was switched into the intact PTH assay performed by the Abbott Architect analyzer, when the CIS BIO assay was no longer available. The Abbott Architect assay was recalibrated on the CIS BIO assay to give identical measurement results.

Statistical analysis

Statistical analysis was performed using the Statistical Product and Services Solutions (SPSS) package version 16.0. The Wilcoxon rank-sum test was used to compare differences between groups (unless otherwise stated). The level of significance was set at $P < 0.05$.

RESULTS

We investigated the effect of low versus high dose cholecalciferol (vitamin D_3) on serum 25(OH)D_3 and PTH levels in HIV-infected subjects. There were 3 study groups: the high-dose cohort (n = 7) received weekly oral cholecalciferol supplementation at a dose of 25000 IU for 2 months, the low-dose treatment arm (n = 9) was given 3 months of 800 IU/day cholecalciferol supplementation orally and the control group (n = 7) received no supplementation and was followed up from spring to summer. The patients' characteristics are presented in Table 1.

	High-dose cohort (n=7)	Low-dose cohort (n=9)	Non-treated cohort (n=7)
Median age (range)	33 (22-46)	39 (24-57)	37 (25-53)
Gender			
Male	3	7	5
Female	4	2	2
Ethnicity			
African	6	5	3
Caucasian	1	4	3
Hispanic	0	0	1

Table 1. Demographic data of the patients

High weekly dose of 25 000 IU cholecalciferol achieve the optimal target serum 25(OH)D_3 concentration in HIV-infected patients

The baseline median serum 25(OH)D_3 and PTH levels in the high-dose cohort (n=7) were 30.0 nmol/L and 7.7 pmol/L respectively. After weekly cholecalciferol supplementation at 25000 IU for 2 months, the median serum 25(OH)D_3 and PTH levels were 138.0 nmol/L and 6.7 pmol/L respectively (Figure 1A). In the low-dose cohort (n = 9), the baseline median serum 25(OH)D_3 and parathyroid hormone (PTH) levels were 23.8 nmol/L and 7.2 pmol/L respectively. After 3 months of 800 IU/day cholecalciferol supplementation, the median

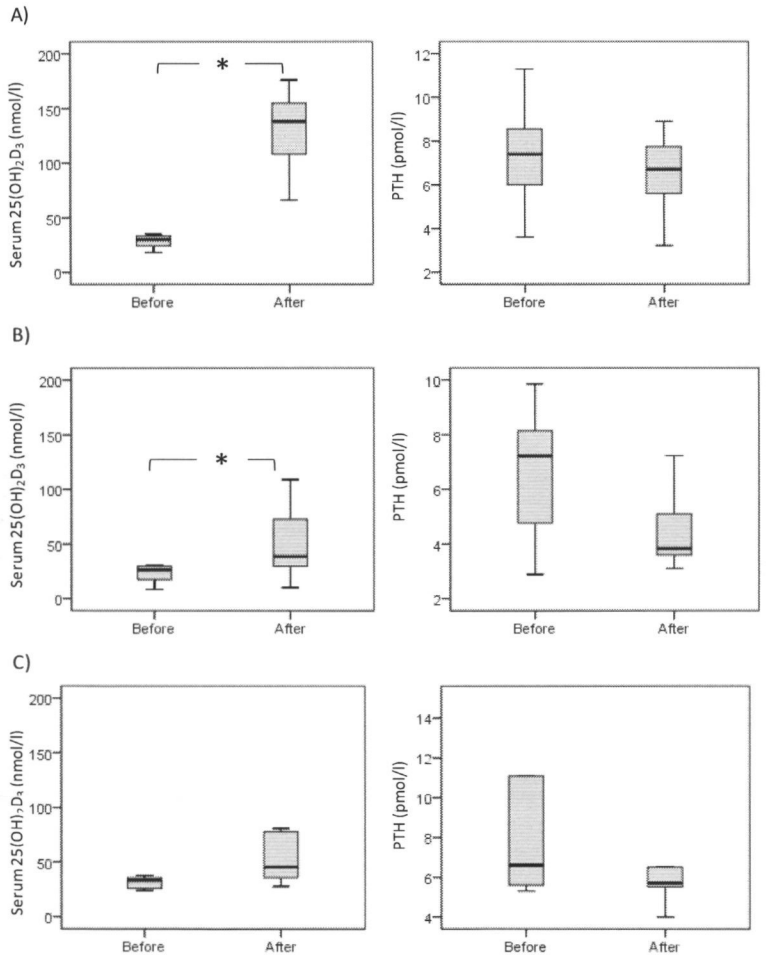

Figure 1. Serum 25(OH)D_3 concentrations and parathyroid hormone (PTH) levels

Serum 25(OH)D_3 and PTH concentrations in A, B) high-dose, C, D) low-dose and E, F) control cohort before and after treatment. The high-dose cohort (n = 7) received a weekly oral dose of 25000 IU cholecalciferol for 2 months. The low-dose study cohort (n = 9) received a daily oral dose of 800 IU cholecalciferol for 3 months. In the control group (n = 7) recruited from February to May and followed through up for 2 to 3 months, no supplementation was administered. *P < 0.05 as compared to baseline.

serum 25(OH)D_3 and PTH levels were 38.7 nmol/L and 3.8 pmol/L respectively (Figure 1B). The optimal target serum 25(OH)D_3 of 75 nmol/L was achieved in 86% (6/7) of the high-dose study cohort but only 2 subjects (22%) in the low-dose treatment group attained this target concentration.

From February to May 2009, seven HIV-infected subjects were recruited and no vitamin D_3 supplementation was given to this group. They were monitored for the effect of seasonal variation of vitamin D_3 status after 2 to 3 months. In spring, the median serum 25(OH)D_3 and PTH levels were 33.0 nmol/L and 6.6 pmol/L respectively and in summer, the median serum 25(OH)D_3 and PTH levels were 48.5 nmol/L and 5.7 pmol/L respectively (Figure 1C).

Modulation in the skin-homing capacity of Treg can only be observed in high dose vitamin D_3 supplementation

In the next set of experiments, we investigated whether vitamin D_3 supplementation can modulate the homing capability of Treg in HIV-infected patients. For this purpose, we evaluated the skin-homing markers, CCR4 and CCR10 as well as gut-homing markers, CCR9 and integrin $\alpha_4\beta_7$ using flow cytometry (Figure 2).

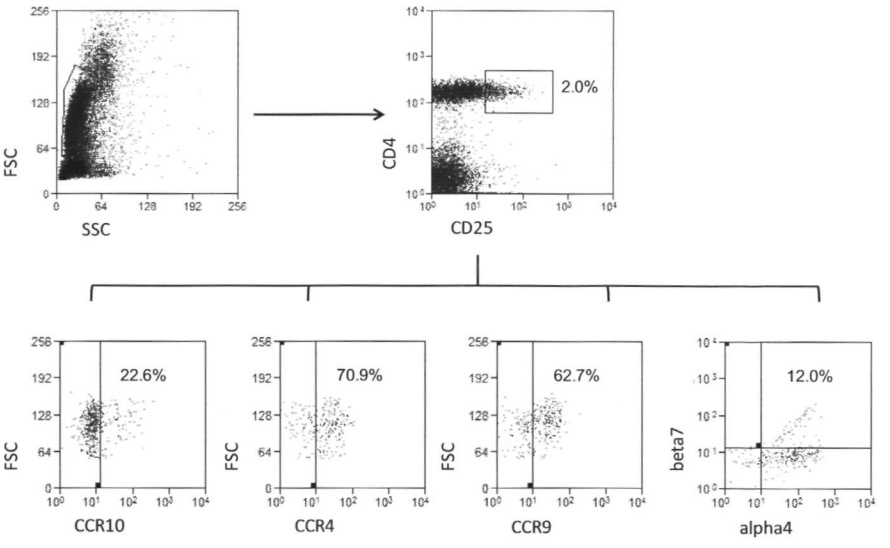

Figure 2. Representative flow cytometry dotplots for homing markers expression in HIV-infected patients treated with high dose cholecalciferol

Gate setting for CCR10, CCR4, CCR9 and integrin $\alpha_4\beta_7$; cells were gated on $CD4^+CD25^{hi}$ regulatory T cells. Dotplots show surface staining for markers analyzed on PBMC.

A significant increase in the CCR10 expression (mean fluorescence intensity, MFI) by Treg was seen in the high-dose cohort after treatment (Figure 3A). In the same study group, a decrease in the skin-homing chemokine receptor, CCR4 expression level (MFI) was observed together with a lower percentage of Tregs expressing CCR4 (Figure 3C). However, in the low-dose treatment group no change in both skin-homing markers could be observed after 3 months (data not shown).

We verified our *in vivo* findings by performing the analysis on regulatory T cells isolated from healthy volunteers and tested the *in vitro* effects of vitamin D_3 using supraphysiological dose of $1,25(OH)_2D_3$. Treg were stimulated with anti-CD3/anti-CD28 mAb-coated microbeads, in the presence of exogenously added rhIL-2 and 100 nM $1,25(OH)_2D_3$ and analyzed after an incubation period of 4 days. The percentage of Tregs expressing CCR10 was significantly increased (Figure 3B). A significantly elevated level of expression (MFI) of CCR10 by Treg was also apparent. On the other hand, there was no significant effect observed for the expression of CCR4 *in vitro* (Figure 3D).

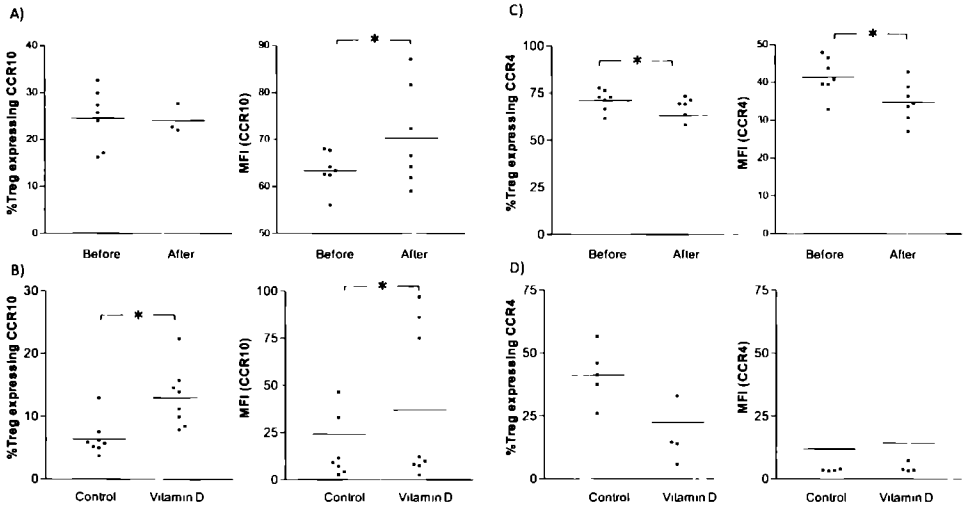

Figure 3. *In vivo* and *in vitro* effects of vitamin D_3 on skin-homing markers CCR10 and CCR4 expression by regulatory T cells

Percentage and level of expression (mean fluorescence intensity, MFI) of skin-homing marker A) CCR10 and C) CCR4 by Treg in high-dose treatment group. PBMC from the study cohort were analyzed for the respective markers using flow cytometry; cells were gated on $CD4^+CD25^{hi}$ Treg. Percentage and level of expression (MFI) of B) CCR10 and D) CCR4 on high purity flowcytometrically sorted-$CD4^+CD25^{hi}CD127^-$ Treg from healthy individuals. Cells were stimulated with anti-CD3/anti-CD28 mAb-coated microbeads in the presence or absence (carrier) of 100 nM $1,25(OH)_2D_3$ for 3-4 days and analyzed for the respective markers using flow cytometry. Data show results from 5 to 6 independent experiments performed with cells obtained from different donors. *$P < 0.05$ as compared to baseline or respective untreated cell culture without the addition of $1,25(OH)_2D_3$.

Vitamin D_3 has no effect on gut-homing markers *in vivo* and *in vitro*

There were no changes in the frequency and expression levels (MFI) of the gut-homing markers, CCR9 and integrin $\alpha_4\beta_7$ with either low daily dose (data not shown) or high-dose pulsed therapy with vitamin D_3 in HIV-infected patients (Figure 4A and 4C). Similarly, we looked at the expression of these markers *in vitro*, using Tregs isolated from healthy volunteers and stimulating these cells with anti-CD3/anti-CD28 mAb-coated microbeads and tested the effect of 100 nM $1,25(OH)_2D_3$. Parallel to our *in vivo* observation, vitamin D_3 has no effect on CCR9 and integrin $\alpha_4\beta_7$ expression *in vitro* (Figure 4B and 4D).

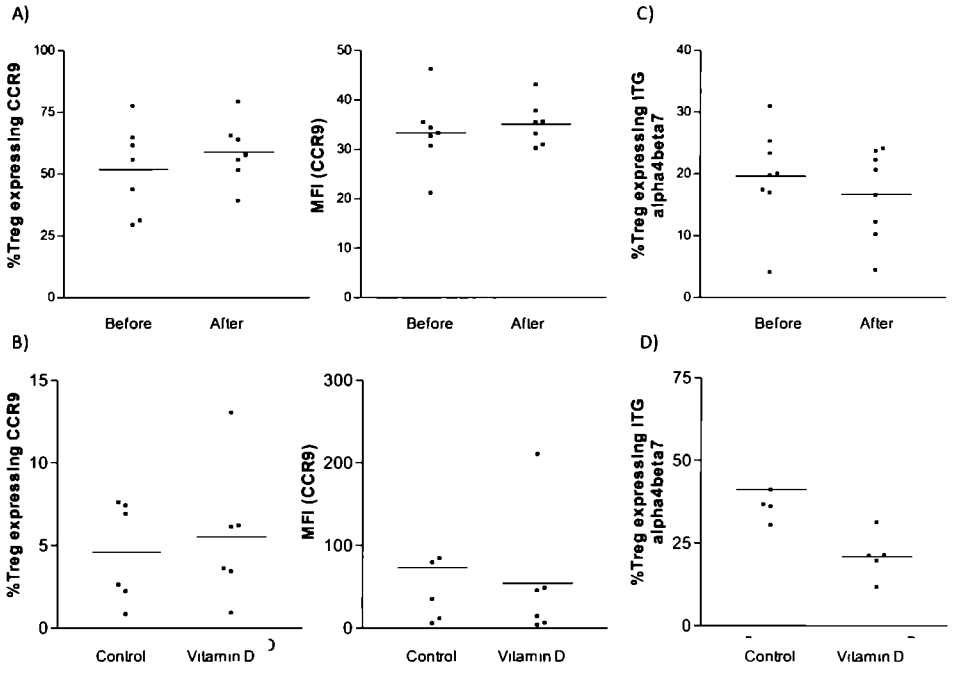

Figure 4. Effects of vitamin D_3 on gut-homing marker CCR9 and integrin $\alpha_4\beta_7$ expression by regulatory T cells *in vivo* and *in vitro*

Percentage of Treg expressing A) CCR9 and C) integrin (ITG) $\alpha_4\beta_7$, and A) level of CCR9 expression (MFI) by Treg in high-dose treatment group. PBMC from the study cohort were analyzed for the respective markers using flow cytometry; cells were gated on $CD4^+CD25^{hi}$ Treg. Frequency of B) CCR9 and D) integrin (ITG) $\alpha_4\beta_7$ expression, and B) level of CCR9 expression (MFI) by $CD4^+CD25^{hi}CD127$ Treg isolated by high purity flowcytometric cell sorting. Cells were stimulated with anti-CD3/anti-CD28 mAb-coated microbeads and incubated in the presence or absence (carrier) of 100 nM $1,25(OH)_2D_3$ for 3-4 days and analyzed for the respective markers using flow cytometry. Data show results from 5 to 6 independent experiments performed with cells obtained from different donors. $^*P < 0.05$ as compared to baseline or respective cell culture without the addition of $1,25(OH)_2D_3$.

DISCUSSION

In this study, we used two different doses of vitamin D_3 supplementation to investigate its effect on regulatory T cell trafficking in patients infected with HIV-1. We employed the standard recommended dose of 800 IU/day (22) versus a high dose regimen of 25000 IU/week cholecalciferol (vitamin D_3) in this study. In the high-dose study cohort, skin-homing marker CCR10 expression on Treg was increased while CCR4 expression and the percentages of CCR4-expressing cells were decreased. On the other hand, there were no notable differences in the expression of either the skin- or gut-homing markers in the low-dose treatment group. The lack of effect seen in the low-dose cohort could be related to the lack of treatment efficacy with a standard dose of 800 IU cholecalciferol per day in this specific patient population. We saw that only 22% (2/9) of the patients in this cohort responded with a significant drop in PTH level and achieve serum 25(OH)D_3 level of >75 nmol/L. This effect is similar to that achieved by UV exposure during summer in the study region (Figure 1C).

High-dose pulsed therapy seems effective in correcting vitamin D_3 deficiency in 86% (6/7) of HIV-infected patients. The median serum 25(OH)D_3 was elevated by 3-fold, from 33.0 nmol/L to 138.0 nmol/L after 2 months. There is limited knowledge on the immunological influence of vitamin D_3 supplementation in HIV-infected patients or the effective dose in this specific group of patients. In a cohort of 56 children (age 6 to 16) randomized to receiving a bi-monthly supplementation of 100,000 IU cholecalciferol (with daily doses of calcium) or placebo, serum 25(OH)D_3 concentrations were higher in the treated group but no differences in CD4 count or viral load over a 12-month period were observed (11). In another study carried out in 40 patients with multiple sclerosis (MS), the efficacy of low dose cholecalciferol (800 IU or less) and high dose ergocalciferol (50,000 IU for 7-10 days and subsequently once or twice weekly) was being compared (23). Parallel to our observation, the low dose cholecalciferol treatment was unable to reverse the deficiency in MS patients while switching the patients to a high dosage of vitamin D_2 resulted in a significant increase in their serum 25(OH)D_3 level. Of note, these patient cohorts share two things in common with ours in terms of geographical location (northern hemisphere) and both have an underlying immunopathology of different nature. The fact that we did not detect any significant differences in chemokine receptors expression by Treg in the low-dose cohort implies that it would be worthwhile using a higher dose to study the immunomodulatory effects of vitamin D_3 in patients with an underlying immune dysfunction. In fact, it has been suggested that a higher 25(OH)D_3 level (>100 nmol/L) may be required to sustain the immune function (24).

Homing receptors are selectively expressed and regulated in different T cells subset (19;25). Skin-homing T cells express CLA and by their binding to vascular E-selectin initiates the T cell rolling interactions on vascular endothelium, the first step in the migration process. Migration to the skin also involves CCR4 for the transition of blood to the dermis and CCR10 from the dermis to the epidermis, and interactions of integrin $\alpha_4\beta_1$ with VCAM and of LFA-1 with ICAM-1. In human T cells, $1,25(OH)_2D_3$ has been reported to down-regulate CLA expression but not CCR4, CCR6 and CCR7 (26). *In vitro*, it has been shown that addition of $1,25(OH)_2D_3$ after cells were activated resulted in an induction of CCR10 and inhibition of CLA expression (27). In the present study, high dose cholecalciferol supplementation in HIV-infected individuals differentially augments the expression of skin-homing markers, CCR4 and CCR10 on Treg. *In vivo* vitamin D_3 up-regulates CCR10 expression and depresses CCR4 expression as well as the frequency of CCR4-expressing Treg. Parallel to our *in vivo* observation, CCR10 expression on Treg was elevated *in vitro* while the effect on CCR4 was not significant. The eventual result could still be a preferential homing of Treg to the skin as influenced by $1,25(OH)_2D_3$. One should consider that even though both CCR10 and CCR4 are implicated in lymphocyte-trafficking to the skin, there may be a certain degree of overlap in the two pathways with either alone being sufficient for skin homing (28). It has been shown that T cell accumulation within the skin was not impaired in CCR4-deficient mice (29;30). Lehtimaki reported that Treg are able to infiltrate the skin of CCR4-/- mice as evident by an increased number of Treg and enhanced Foxp3 mRNA in an oxazolone-induced inflamed skin model (29). Hence even though we observed a depressed CCR4 expression alongside an elevated CCR10 expression on Treg in HIV-infected subjects supplemented with high weekly dose of cholecalciferol, it is tempting to speculate that the net effect of vitamin D_3 may be a preferential homing of Treg to the skin.

The integrin $\alpha_4\beta_7$ and CCR9 are expressed by T cells which migrate to the gut. As previously described, retinoic acid and vitamin D_3 which are selectively produced in the gut and skin respectively, differentially modulate the expression of gut- and skin- homing markers (31). Retinoic acid has been associated with up-regulation of CCR9 and integrin $\alpha_4\beta_7$ expression while reducing the expression of CCR10 on T cells. On the other hand, $1,25(OH)_2D_3$ does not affect the gut-homing markers but increased CCR10 expression (31). Here, we did not observe any *in vivo* or *in vitro* effect by $1,25(OH)_2D_3$ on the expression of CCR9 and integrin $\alpha_4\beta_7$. Of note, it has been demonstrated that the HIV-1 envelope protein gp120 binds to $\alpha_4\beta_7$ (32). As a result, $CD4^+CCR5^{hi}\alpha_4\beta_7^{hi}$ T cells are highly susceptible to infection by HIV-1 and gp120 engagement of $\alpha_4\beta_7$ is thought to facilitate infection at the point of transmission.

In light of the importance of vitamin D_3 in regulating the immune function, interventions to improve vitamin D_3 status in HIV-infected patients continues to warrant attention. Our findings serve as a caution to clinicians that treatment with a standard dose of 800 IU per day cholecalciferol may be inadequate to reverse the deficiency in HIV-infected individuals or to serve any immunomodulatory effects. The effect from a high weekly pulsed -dose therapy with vitamin D_3 in HIV-infected individuals is suggestive of induction of skin-homing potential on Treg.

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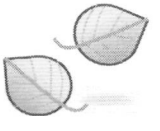
Chapter 9

Summary and Discussion



SUMMARY AND DISCUSSION

Since the uncovering of an extra-renal source of 1α-hydroxylase (1) and the expression of vitamin D receptor (VDR) by immune cells (2) almost 30 years ago, $1,25(OH)_2D_3$ has displayed a far-reaching effect on human health. However, it was not until recent years that the physiological relevance of vitamin D_3-mediated immunity became more apparent and consequently the pursuit in vitamin D research is ever more intense. Annual citations in the PubMed database on vitamin D in 2010 were approximately 3600, doubling the volume as compared to a decade ago.

In this thesis, we explored the role of vitamin D_3 from three angles: its interaction with pathogens, the impact of its physiological variation on immunity and disease, and finally its immunomodulatory effect on the regulatory arm of the immune response. The observations and results of the studies presented in Chapter 2 through Chapter 8 are briefly described and further discussed below.

In **Chapter 2** of this thesis, we summarized the current knowledge on the role of vitamin D_3 as regulator of the immune system with specific reference to protective mechanism against pathogens. The net result of $1,25(OH)_2D_3$ action on T cell is skewing towards regulatory T cell (Treg) differentiation and away from T helper (Th)1 and Th17 polarization. This is essential in curtailing an excessive and unchecked proinflammatory host response in certain situations such as sepsis and chronic infections. The recent discovery of its ability to promote production of the antimicrobial peptide cathelicidin underlines a protective mechanism by vitamin D_3 in infections. Cathelicidin enhances microbial killing through disruption of the bacterial membrane and can also activate other antimicrobial pathways within infected cells. Over the past two decades, there is growing epidemiological evidence that suboptimal vitamin D_3 status is correlated with the prevalence of many diseases not previously associated with this nutrient. Higher vitamin D_3 status is positively associated with increased protection from infection with influenza, tuberculosis (TB) and respiratory tract viruses. However, such data amounts only to circumstantial evidence. As low vitamin D_3 levels may be itself a consequence of the disease process, evidence for vitamin D_3 affecting disease pathogenesis must come from interventional studies. We discussed the clinical data from randomized placebo-controlled trials (RCT) in order to identify its therapeutic potential in infections such as TB, viral influenza and human immunodeficiency virus (HIV) infection.

From the studies we reviewed, vitamin D_3 supplementation can potentially modify the clinical course and susceptibility to tuberculosis, and decrease the prevalence of viral respiratory tract infection. However, well-designed RCT are needed to elucidate the optimal vitamin D_3 doses as adjunctive therapy.

The earliest recorded use of vitamin D was in 1849 when British physician, Dr Williams used cod liver oil to treat tuberculosis (known as phthisis at that time; defined as a disease characterized by wasting) (3). Although this is no longer the standard of care, the application of vitamin D_3 in TB has since been re-visited most extensively amongst other infectious diseases. There have been numerous RCT (4-6) reported in recent years, as well as a comprehensive illustration of its underlying immunomodulatory mechanism (7). In **Chapter 3**, we demonstrated that $1,25(OH)_2D_3$ down-regulates the proinflammatory cytokine response *in vitro* when human leukocytes are stimulated with *M. tuberculosis*. Upon further evaluation, this appeared to be a consequence of suppression of Toll-like receptor (TLR) 2, TLR4, Dectin-1 and mannose receptor expression. On the other hand, production of the protective antimicrobial peptide cathelicidin was enhanced by vitamin D_3. Even though Th1 cytokines are vital for an effective immunity against *M. tuberculosis*, a disproportionate proinflammatory response often leads to excessive tissue damage (8). Taken together, these two effects complement each other by suppressing prolonged inflammation associated with TB and thereby prevent immune-mediated tissue damage, while offering protection through the antibacterial effect of vitamin D_3-induced cathelicidin. Moving forward, strategies to manipulate vitamin D_3 therapy such that an antimicrobial effect can be achieved in TB without an excessive proinflammatory response would be an uphill task. Further studies are warranted to optimize this *in vitro* model and translate it into *in vivo* studies and to titrate the vitamin D dosages so that it can attain the desired clinical outcome. Moreover, it is reasonable to hypothesize that this 'dual action' of vitamin D_3 may be of value in other chronic infections such as *Candida albicans*, where cathelicidin seems to exert a protective role (9).

Our knowledge on the potential influence of vitamin D_3 on fungal infection is limited (10), while vitamin D_3 has been tested extensively as a preventive agent in viral influenza (11-13) and tuberculosis (4;6;14;15). In **Chapter 4**, we employed an *in vitro Candida albicans* model and showed that $1,25(OH)_2D_3$ down-modulates the proinflammatory cytokine response through suppression of TLR2, TLR4, Dectin-1 and mannose receptor expression. We have illustrated a novel role for $1,25(OH)_2D_3$ in the host innate response to *C. albicans*, but its

clinical relevance in *C. albicans* and other fungal infection merits further investigation. In line with the observations from Chapters 3 and 4, the challenge is to characterize and analyze the effects of vitamin D_3 on the cytokine profile in these patients, from acute infection through the persistent phase. Such information will provide insights on how best to tailor vitamin D_3 therapy to exert an anti-inflammatory effect without compromising antimicrobial activities. However, it is prudent to consider the possible effects that antimicrobial therapy may have on vitamin D_3 metabolism. The enzymes involved in vitamin D_3 metabolism, such as 1α-hydroxylase, 25-hydroxylase and 24-hydroxylase, are part of the cytochrome (CYP)450 enzyme system. Many antimycobacterial, antifungal and antiretroviral drugs are known to induce or inhibit the CYP450 iso-enzymes so there is a potential risk for interaction with the metabolism of vitamin D_3 and ultimately the systemic or local vitamin D_3 concentration (16).

With an understanding that $1,25(OH)_2D_3$ modulates proinflammatory cytokine production during infection *in vitro*, and before we validate these findings *in vivo*, it is worthwhile to establish how physiological variation in vitamin D_3 status can modulate the immune response. Seasonal variation in vitamin D_3 status with reduced sunlight exposure during certain periods of the year is thought to be responsible for the high prevalence of vitamin D_3 insufficiency among populations residing at higher latitudes. Not surprisingly, a positive latitude gradient for disease prevalence of certain cancers (17) as well as autoimmune diseases such as multiple sclerosis (MS) (18) and insulin-dependent diabetes mellitus (19) have been recorded. In **Chapters 5 and 6**, we presented novel findings on how seasonal variation in serum $25(OH)D_3$ concentrations can influence the innate and adaptive immunity in healthy adults. In **Chapter 5**, a significantly elevated serum $25(OH)D_3$ concentration during summer suppressed the proinflammatory cytokine response in healthy individuals. This effect was found to be related to a diminished expression of TLR2 and TLR4. In **Chapter 6**, we found seasonal changes in the peripheral T cell composition, phenotype and function. It would be useful to relate some of these observations with previous epidemiological findings on the prevalence of autoimmune diseases and infections, which coincide with the seasonal variation in vitamin D_3 status. Therefore the appropriate course of action would be to verify these results in larger population-based studies, as well as in patient cohorts. Noteworthy, it has been suggested that a serological response to influenza vaccine is more frequent among prostate cancer patients with higher serum $25(OH)D_3$ levels (20). The season of birth can influence the risk of developing MS, and children born to mothers who were exposed to low levels of ultraviolet radiation in the first trimester have an increased risk of developing the disease later in life (21). The proposition that vaccine

responses may vary with the season and that the severity of specific autoimmune diseases may improve during summer merits further investigation. Ultimately, the gain of this type of information may improve decision-making in public health policies on vitamin D_3 supplementation.

The final part of this thesis addressed the immunological impact of $1,25(OH)_2D_3$ on regulatory T cells. Vitamin D_3 is known to manipulate the differentiation and maturation of dendritic cells, inhibit T cell proliferation and alter cytokine production. Vitamin D_3 inhibits the generation of Th1 and Th17 responses, while it induces Tregs by rendering dendritic cells tolerogenic. However, its immediate effect on human naturally-occurring Treg is less apparent. In **Chapter 7**, we addressed the effects of $1,25(OH)_2D_3$ on Tregs in the absence of antigen presenting cells. *In vitro* $1,25(OH)_2D_3$ could directly inhibit the proliferation of human naturally-occurring Tregs with limited effects on cytokine production, while preserving its suppressive function. One striking observation was that Treg proliferation was more susceptible to the suppressive effect of $1,25(OH)_2D_3$ as compared to conventional T cell (Tconv). Our experiment revealed that Treg, similarly to Tconv, express VDR, and that the expression is induced upon T cell receptor (TCR) stimulation. This highlights the fact that $1,25(OH)_2D_3$ can affect Tregs directly. Therefore, the interplay between VDR and TCR signaling (22) in Treg is pivotal in outlining the mechanistic role of $1,25(OH)_2D_3$ and deserves further attention.

Human immunodeficiency virus infection is a disease of $CD4^+$ T cells pathology. To study the impact of $1,25(OH)_2D_3$ on Treg during disease state, we looked at how cholecalciferol (vitamin D_3) supplementation can modify Treg phenotype in HIV-infected individuals. Reported as a pilot study in **Chapter 7**, reduced Treg numbers were seen in parallel with an elevated serum $25(OH)D_3$ levels when vitamin D_3 deficient HIV-infected patients were supplemented with 2000 IU cholecalciferol daily. Apart from suppressed proliferation, we postulated the possibility of redistribution of circulating Tregs and examined how $1,25(OH)_2D_3$ may augment their trafficking potential *in vivo*. In **Chapter 8**, we illustrated that high-dose pulsed therapy with 25 000 IU cholecalciferol supplementation, differentially modulated skin-homing markers on Tregs with an increased level of CCR10 expression and reduced CCR4 expression. A significant finding was that cholecalciferol supplementation administered at 25 000 IU weekly promoted restoration of serum $25(OH)D_3$ levels more effectively as compared to a standard dose of 800 IU daily. This brings to mind the fundamental question of defining an adequate vitamin D_3 dose for immunomodulatory

activities. Current target serum 25(OH)D_3 concentration and dosage recommendation for vitamin D_3 are governed by its therapeutic application in bone and calcium homeostasis (23). The dose required for achieving the same serum 25(OH)D_3 concentration in patients with an underlying immune-mediated pathology may differ from healthy individuals. Furthermore, the optimal dosage as immunotherapy may differ among various diseases and remains to be determined.

Vitamin D_3 displays a multitude of activities and is known to be involved in a variety of diseases. It influences process such as immune regulation, host defense, inflammation, and cell proliferation. The findings in this thesis offer new perspectives on its immunological effects, as we seek to learn more about its mechanism of actions in various conditions and to translate it into clinical application. Having said that, several hurdles must be overcome to validate the benefits of vitamin D_3 therapeutics. Many of its mechanisms in human pathology are not clearly elucidated and the molecular pathways involved are likely difficult to identify because vitamin D_3 influences in parallel a variety of biological processes. The versatility of vitamin D_3 also makes it challenging to determine the optimal dose needed to confer a beneficial outcome in different disease states.

Epidemiological data suggest that the association between vitamin D_3 deficiency and various pathologies is widespread, but conclusive data from interventional studies are missing for many diseases. Moving forward, randomized placebo-controlled trials taking into account variation in ethnicity, geographical location and seasonal variability in vitamin D_3 status are needed to effectively decipher its therapeutic value. In addition, we propose that at least a few major questions need to be addressed: the dose to be administered, the time frame for potential benefits, the serum 25(OH)D_3 concentrations to be sought for maximal clinical efficacy, the stage of disease most susceptible to this intervention, and the role of genetic variation in controlling responses to vitamin D_3. The assessment of immunological functions would be of value in correlating with clinical outcomes. As much as we have advanced in our discovery of the non-classical roles of vitamin D_3, new challenges present as we continue to unravel its clinical roles, in the hope of rejoicing in the 're-birth' of vitamin D_3 when we are able to translate this knowledge into clinical benefits.

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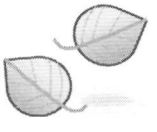
Nederlandse Samenvatting en Discussie



Nederlandse Samenvatting en Discussie

Sinds de ontdekking dat er ook buiten de nier 1α-hydroxylase bronnen zijn (1) en er bekend werd dat ook immuuncellen de vitamine D receptor (VDR) tot expressie kunnen brengen (2), bijna 30 jaar geleden, heeft $1,25(OH)_2D_3$ een wijdverbreid effect gehad op de volksgezondheid. Met name de afgelopen jaren werd de relevantie van vitamine D_3 gemedieerde immuniteit steeds duidelijker en is er groeiende aandacht voor vitamine D. Jaarlijkse citaties in de database van PubMed op de zoekopdracht 'vitamine D' in 2010 leverde ongeveer 3600 hits op, dit is een verdubbeling ten opzichte van een decennium geleden.

In de studies beschreven in dit proefschrift is de rol van vitamine D_3 bestudeerd vanuit drie invalshoeken: de interactie met ziekteverwekkers, de impact van de fysiologische variatie op immuniteit en ziekte en ten slotte het immuunmodulerende effect op de regulerende arm van de immuunrespons. De waarnemingen en de resultaten van de studies zijn gepresenteerd in hoofdstuk 2 tot en met hoofdstuk 8 van dit proefschrift. Hieronder volgt een korte beschrijving van de inhoud:

In **hoofdstuk 2** van dit proefschrift wordt de huidige kennis over de rol van vitamine D_3 als regulator van het immuun systeem samengevat, met specifieke verwijzing naar beschermend mechanisme tegen ziekteverwekkers. Het netto resultaat van het effect $1,25(OH)_2D_3$ op T cellen *in vitro* is de sturing richting regulatoire T cel (Treg) differentiatie en weg van T helper (Th)1 en Th17 polarisatie. Dit is essentieel om een ontspoorde ontstekingsreactie in bepaalde situaties zoals sepsis en chronische infecties onder controle te houden. De recente ontdekking dat vitamine D_3 de productie van het antimicrobiële peptide cathelicidin kan bevorderen onderstreept een beschermend mechanisme door vitamine D_3 in infecties. Cathelicidin verbetert microbiële killing door het verstoren van het bacteriële membraan en kan ook andere antimicrobiële routes in geïnfecteerde cellen activeren. In de afgelopen twee decennia is er steeds meer epidemiologisch bewijs dat suboptimaal vitamine D_3 status is gecorreleerd met de prevalentie van vele ziekten, die eerder niet in verband werden gebracht met vitamine D_3. Een hoog vitamine D_3 waarde is positief geassocieerd met een betere bescherming tegen besmetting met influenza, tuberculose (TB) en virussen aan de luchtwegen. Dit soort studies zijn slechts indirect bewijs. Lage vitamine D_3 waardes zouden ook een gevolg kunnen zijn van het ziekteproces. Om experimenteel te bewijzen dat vitamine D_3 inderdaad de pathogenese van een ziekte

kan beïnvloeden dienen interventie studies te worden uitgevoerd. Wij bediscussiëren de klinische gegevens uit gerandomiseerde placebo-gecontroleerde studies (RCT) om het therapeutisch effect vast te stellen in infecties zoals tuberculose, virale influenza en humaan immuundeficiënt virus (HIV) infectie. Uit de studies die we bestudeerden, bleek dat vitamine D_3 suppletie het ziekteverloop en de gevoeligheid voor tuberculose bepalen en de prevalentie van virale luchtweginfecties verminderen. Goed ontworpen RCT zijn echter nodig om de optimale vitamine D_3 doses die geschikt is als therapeutisch adjuvant op te helderen.

Het eerste geregistreerde gebruik van vitamine D was in 1849 toen de Britse arts, Dr Williams levertraan gebruikte voor de behandeling van tuberculose, op dat moment bekend als phthisis; gedefinieerd als een ziekte die wordt gekenmerkt door wegkwijnen (3). Hoewel dit niet meer de standaard benadeling is, heeft de toepassing van vitamine D_3 in TB zich volop uitgebreid voor de behandeling van andere besmettelijke ziekten. Er zijn talrijke RCT (4-6) gemeld in de afgelopen jaren, evenals een uitgebreide illustratie van de onderliggende immuunmodulerende mechanismen (7). In **hoofdstuk 3**, laten we zien dat $1,25(OH)_2D_3$ de proinflammatoire cytokine reactie onderdrukt *in vitro,* wanneer humane leukocyten worden gestimuleerd met *M. tuberculosis*. Na verdere evaluatie bleek dit een gevolg te zijn van de onderdrukking van Toll-like receptor (TLR) 2, TLR4, Dectin-1 en mannose receptor expressie. Tevens vonden we dat vitamine D_3 de productie van het beschermende antimicrobiële peptide cathelicidin verhoogde. Hoewel Th1 cytokines van vitaal belang zijn voor een effectieve immuniteit tegen *M. tuberculosis*, leidt een ongecontroleerde proinflammatoire reactie vaak tot weefselschade (8). Samengevat, deze twee effecten vullen elkaar aan, enerzijds het onderdrukken van een langdurige ontsteking geassocieerd met TB en daarmee het voorkomen van immuun-gemedieerde weefselschade, anderzijds bescherming door de antibacteriële werking van het door vitamine D_3-geïnduceerde cathelicidin. Voor de toekomst ligt er de uitdagende taak om vitamine D_3 therapie te manipuleren zodanig dat er een antimicrobiële werking kan worden bereikt in TB zonder overmatige ontstekingsreactie. Verdere studies zijn gerechtvaardigd om het *in vitro* model te optimaliseren en te vertalen naar *in vivo* onderzoek en de doseringen van vitamine D_3 zodanig te titreren, zodat het de gewenste klinische resultaten kan bereiken. Bovendien, lijkt het redelijk te veronderstellen dat de 'dubbele actie' van vitamine D_3 van waarde kan zijn in andere chronische infecties zoals *Candida albicans*, waar cathelicidin ook een beschermende rol lijkt te hebben (9).

Onze kennis over de potentiële invloed van vitamine D_3 op schimmelinfectie is beperkt (10) terwijl vitamine D_3 uitgebreid is getest als een preventief middel bij virale influenza (11-13) en tuberculose (4;6;14;15). In **hoofdstuk 4**, tonen we in een *in vitro Candida albicans* model aan dat $1,25(OH)_2D_3$ de proinflammatoire cytokine respons verminderd door middel van onderdrukking van TLR2, TLR4, Dectin-1 en mannose receptor expressie. We hebben een nieuwe rol voor $1,25(OH)_2D_3$ bloot gelegd in de aangeboren immuunreactie tegen *C. albicans*. Echter, de klinische relevantie in *C. albicans* en andere schimmelinfecties vereist verder onderzoek. In overeenstemming met de gerapporteerd bevindingen in de hoofdstukken 3 en 4, is het nu de uitdaging om de gevolgen van vitamine D_3 op het cytokine profiel in patiënten, van acute infectie tot aan de persisterend fase te karakteriseren en te analyseren. Dergelijke informatie zal inzicht geven over de optimale wijze van vitamine D_3 therapie, met als doel ontsteking te remmen zonder verlies van antimicrobiële werking. Het is wel van belang aandacht te besteden aan de mogelijke gevolgen die antimicrobiële therapie op het vitamine D_3 metabolisme kan hebben. De enzymen die betrokken zijn bij vitamine D_3 metabolisme, zoals 1α-hydroxylase, 25-hydroxylase en 24-hydroxylase, maken deel uit van de cytochroom P450 (CYP450) enzymsysteem. Van veel antimycobacteriële, schimmeldodende en antiretrovirale geneesmiddelen is bekend dat zij de CYP450 iso-enzymen remmen, dus er is een potentieel risico voor interactie met het metabolisme van vitamine D_3 en uiteindelijk de lokale of systemische vitamine D_3 concentratie (16).

Nu we weten dat $1,25(OH)_2D_3$ de productie van proinflammatoire cytokines in ons *in vitro* infectie model moduleert, is het van belang deze bevindingen *in vivo* valideren. Echter voor het zover is, is het van belang om vast te stellen hoe fysiologische variatie de vitamine D_3 status en de immuunreactie kan moduleren. Seizoensvariatie in vitamine D_3 status met verminderde zonlicht gedurende bepaalde periodes van het jaar wordt beschouwd als verantwoordelijk voor de hoge prevalentie van vitamine D_3 insufficiëntie onder de bevolking woonachtig op hogere breedtegraden. Een positieve correlatie tussen breedtegraad en ziekte prevalentie van bepaalde kankers (17) en immuunziekten zoals multiple sclerose (MS) (18) en insuline-afhankelijke diabetes mellitus (19) is geconstateerd. In de **hoofdstukken 5 en 6**, presenteren we nieuwe bevindingen hoe seizoensvariatie de serum $25(OH)D_3$ concentraties en de aangeboren en adaptieve immuniteit bij gezonde volwassenen kunnen beïnvloeden. In **hoofdstuk 5** tonen we aan dat een aanzienlijk verhoogde $25(OH)D_3$ serumconcentratie tijdens de zomer, proinflammatoire cytokine reactie in gezonde individuen onderdrukt. Dit effect wordt gerelateerd aan een verminderde expressie van TLR2 en TLR4. In **hoofdstuk 6** vonden we seizoensgebonden veranderingen in de perifere T cel samenstelling, fenotype en functie. Het is waardevol om enkele van deze bevindingen te

relateren aan eerder resultaten van epidemiologisch onderzoek naar de prevalentie van auto-immuun ziekten en infecties, die mogelijk met de seizoensvariatie in vitamine D_3 status samenvallen. Daarom zou het goed zijn om deze resultaten te controleren in grotere bevolking gebaseerde studies, evenals in patiënt cohorten. Het is opmerkelijk dat er is gesuggereerd dat een serologische reactie op griepvaccin vaker onder prostaatkanker patiënten met hogere serum $25(OH)D_3$ niveaus voorkomt (20). Het seizoen van geboorte kan het risico van het ontwikkelen van MS beïnvloeden en kinderen van moeders die werden blootgesteld aan lage niveaus van ultraviolette straling in het eerste trimester hebben een verhoogd risico van het ontwikkelen van de ziekte later in het leven (21). De stelling dat vaccinatie reacties kunnen variëren met het seizoen en dat de ernst van de specifieke auto-immune ziekten tijdens de zomer verbeteren, verdient verder onderzoek. Uiteindelijk, zal deze informatie besluitvorming verbeteren in het volksgezondheidsbeleid met betrekking to vitamine D_3 suppletie.

Het laatste deel van dit proefschrift is gericht op het immunologische effect van $1,25(OH)_2D_3$ op regulatoire T cellen. Van vitamine D_3 is bekend dat het de differentiatie en de rijping van dendritische cellen manipuleert, de T cel proliferatie remt en cytokine productie wijzigt. Vitamine D_3 remt de generatie van Th1 en Th17 reacties en induceert Treg op een indirecte wijze doordat vitamine D_3 tolerogene dendritische cellen induceert. Het directe effect van vitamine D_3 op natuurlijk voorkomende Tregs is echter minder duidelijk. In **hoofdstuk 7** beschrijven we de effecten van $1,25(OH)_2D_3$ op Tregs in de afwezigheid van antigeen-presenterende cellen. *In vitro* kan $1,25(OH)_2D_3$ direct de proliferatie van humaan natuurlijk voorkomende Tregs beïnvloeden met beperkte effecten op cytokine productie en het behoud van de onderdrukkende functie remmen. Een opvallende observatie was dat Treg proliferatie meer vatbaar was voor het onderdrukkende effect van $1,25(OH)_2D_3$ in vergelijking tot conventionele T cellen (Tconv). Uit ons experimenten bleek dat in Treg, op dezelfde manier als in Tconv, de expressie van de VDR kon worden geïnduceerd door T cel receptor (TCR) stimulatie. Dit wijst erop dat $1,25(OH)_2D_3$ rechtstreeks Tregs kan beïnvloeden. Daarom is het samenspel tussen de VDR en TCR signalering (22) in Treg cruciaal in de mechanistische rol van $1,25(OH)_2D_3$ en verdient dit nadere aandacht.

Human immunodeficiency virusinfectie is een ziekte van $CD4^+$ T cellen. Om de impact van $1,25(OH)_2D_3$ te bestuderen op Treg tijdens ziekte, hebben we bestudeerd hoe cholecalciferol (vitamine D_3) suppletie het Treg fenotype in HIV-geïnfecteerde individuen beïnvloedt. Zoals gerapporteerd als een pilot-studie in **hoofdstuk 7**, vonden wij verminderde absolute

aantallen Treg in het perifere bloed, en daarnaast vonden we een verhoogde serum $25(OH)D_3$ niveau, wanneer vitamine D_3 deficiënte HIV-geïnfecteerde patiënten dagelijks werden gesupplementeerd met 2000 IU cholecalciferol. Naast de verminderde Treg proliferatie *in vivo*, hebben de mogelijkheid getoetst of $1,25(OH)_2D_3$ de distributie van circulerende Treg kan beïnvloeden *in vivo* door een verandering in migratie marker expressie patroon. In **hoofdstuk 8**, tonen we aan dat een hoge dosis pulse therapie met 25000 IU cholecalciferol suppletie, de expressie huid-'homing' markers op Tregs differentieel moduleert, met een verhoogd level van CCR10 expressie en verminderd CCR4 expressie. Een belangrijke bevinding was dat wanneer cholecalciferol suppletie van 25000 IU wekelijks toegediend wordt, dit het serum $25(OH)D_3$ niveau effectiever bevorderd in vergelijking met een dagelijks standaard dosis van 800 IU. Dit doet denken aan de fundamentele vraag van het definiëren van een voldoende vitamine D_3 dosis voor immuun modulerende activiteiten. De huidige doelstelling van $25(OH)D_3$ serumconcentratie en de aanbevolen dosering voor vitamine D_3 worden beheerst door de therapeutische toepassing ervan in bot en calcium-homeostase (23). De dosis die nodig is voor het bereiken van dezelfde serum $25(OH)D_3$ concentratie bij patiënten met een onderliggende immuun-gemedieerde pathologie wijkt af van gezonde individuen. Bovendien, de optimale dosering als immunotherapie verschilt tussen de verschillende ziekten en zal moeten worden bepaald.

Vitamine D_3 leidt tot een uiteenlopende hoeveelheid biologische effecten en is betrokken bij diverse ziekten. Het beïnvloedt processen zoals immuunregulatie, host defense, ontsteking en celproliferatie. De bevindingen in dit proefschrift bieden nieuwe perspectieven voor het moduleren van immunologische effecten, we proberen meer te leren over het mechanisme onder verschillende omstandigheden en dit te vertalen naar klinische toepassingen. Dit gezegd hebbend, er moeten eerst nog verschillende hindernissen worden overwonnen om de voordelen van vitamine D_3 therapie te valideren. Veel van de mechanismen in menselijk pathologie zijn nog niet duidelijk en de moleculaire pathways die betrokken zijn, zijn nog maar amper geïdentificeerd, vooral omdat vitamine D_3 een verscheidenheid aan biologische processen beïnvloedt. De veelzijdigheid van vitamine D_3 maakt het uitdagend om de optimale dosis te bepalen die nodig zijn in andere staat van ziekte.

Epidemiologische gegevens suggereren dat de associatie tussen vitamine D_3-deficiëntie en verschillende pathologieën wijdverbreid is, maar afdoende gegevens uit interventionele studies voor vele ziekten ontbreken. Wat we nodig hebben zijn gerandomiseerde placebo-gecontroleerde studies die rekening houden met variatie in etniciteit, geografische locatie en

seizoensgebonden variabiliteit in vitamine D_3 status om daarna effectief de therapeutische waarde te ontcijferen. Bovendien stellen wij voor dat ten minste een paar belangrijke vraagstukken moeten worden aangepakt: de dosis die moet worden toegediend, het tijdschema van toediening, de serum $25(OH)D_3$ concentraties die nodig zijn voor maximale klinische werkzaamheid, het stadium van de ziekte dat het meest vatbaar is voor deze interventie en de rol van genetische variatie in het controleren van reacties op vitamine D_3. Het analyseren van immunologische functies is van groot belang in correlatie met de klinische resultaten. Hoeveel we ook al hebben bereikt in het blootleggen van de "niet-klassieke rol" van vitamine D_3, nieuwe uitdagingen presenteren zich als we doorgaan met het ontrafelen van de klinische relevantie van deze stof. Tezamen maakt dat, dat we kunnen uitkijken naar een 'wedergeboorte' van vitamine D_3, zeker wanneer wij in staat blijken om deze kennis te vertalen naar een verbetering van klinische protocollen en behandeling.

VERWIJZINGEN

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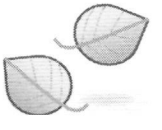
Dankwoord

List of Publications

Curriculum Vitae



Last but not least, I present the most challenging 'chapter' of my thesis. It took me the longest time because there are so many emotions, and some of which are pretty difficult to pen down. With my sincere heart, I would like to convey my deepest gratitude to all you wonderful people...

In spring 2007, I packed my bag and came along with my husband Louis, to Nijmegen. Louis was eager to find me a job while we spend the next couple of years here in this quaint town. Through Prof Mihai Netea, I was most fortunate to have met Prof André van der Ven and that was how this journey begins... ... Prof André van der Ven, you are one person whom I am most indebted to. Without any prior knowledge about who I am or what I can do, you did not hesitate to give me a chance to work in your team. To date, this is one kind act that has been deeply engraved in my heart and I will be eternally grateful to you. You have also been very concerned about my well-being as well as that of Louis and we are truly honoured to have known you.

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heel hartelyk bedankt

Claire

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Claire, Ai Leng Khoo was born in Singapore on 27th March 1978. She graduated from the National University of Singapore in 2001 with a first-class honours degree in Bachelor of Science (Pharmacy). Upon completing her internship in 2002, she continued to serve in the National University Hospital, Singapore as a pharmacist with an interest in critical care and renal medicine. In 2005, she joined Tan Tock Seng Hospital, Singapore and pursued her interest in critical care medicine. In spring 2007, she left for Nijmegen and was presented with the opportunity to study the immunomodulatory role of vitamin D_3 at the Radboud University Nijmegen Medical Center under the mentorship of Prof. dr. Irma Joosten, Prof. dr. André van der Ven, Prof. dr. Mihai Netea and Dr. Hans Koenen. Currently she is working at the National Healthcare Group, Pharmacy and Therapeutics Office, Singapore.

Claire is married to Louis Chai and they have two lovely sons, Clement (born in 2009, Nijmegen) and Damian (born in 2011, Singapore).